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CONTENTS.

PURVES, H. D.—STUDIES ON EXPERIMENTAL GOITRE. IV. THE EFFECT OF DI-IODOTYROSINE AND THYROXINE ON THE GOITROGENIC ACTION OF BRASSICA SEEDS . 171

GRIESBACH, W. E., AND PURVES, H. D.—STUDIES ON EXPERIMENTAL GOITRE. V. PITUITARY FUNCTION IN RELATION TO GOITROGENESIS AND THYROIDECTOMY . 174

GRIESBACH, W. E., AND PURVES, H. D.—THE ASSAY OF THYROTROPIC ACTIVITY BY THE CELL HEIGHT RESPONSE IN GUINEA-PIGS 185

WHITEHEAD, V. I. E.—BASAL METABOLISM OF THE ALBINO RAT FED ON GOITROGENIC DIET 192

FELL, H. B., AND DANIELLI, J. F.—THE ENZYMES OF HEALING WOUNDS. I. THE DISTRIBUTION OF ALKALINE PHOSPHOMONOESTERASE IN EXPERIMENTAL WOUNDS AND BURNS IN THE RAT 196

MCILWAIN, H.—NUTRITIONAL STUDIES OF BACTERIAL VARIATION. I. RESISTANCE TO PANTOYLTAURINE IN NATURALLY-OCCURRING AND EXPERIMENTALLY PREPARED STRAINS 203

MCILWAIN, H.—NUTRITIONAL STUDIES OF BACTERIAL VARIATION. II. THE DERIVATION OF DRUG-RESISTANT STRAINS IN THE ABSENCE OF ANY INHIBITOR . . 212

HOPE SIMPSON, R. E.—CRITERIA OF LIFE AND DEATH IN HUMAN PUS CELLS .. 218

DMOCHOWSKI, L., AND GYE, W. E.—INFLUENCE OF CROSS-SUCKLING ON THE INCIDENCE OF MAMMARY CANCER IN HIGH AND LOW-CANCER STRAINS OF MICE . . 223

SURVEY OF PAPERS i

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STUDIES ON EXPERIMENTAL GOITRE. IV. THE EFFECT OF DI-IODOTYROSINE AND THYROXINE ON THE GOITROGENIC ACTION OF BRASSICA SEEDS.

H. D. PURVES.

From the Thyroid Research Department of the New Zealand Medical Research Council, Otago University, Dunedin, N.Z.

Received for publication August 17, 1943.

In a previous publication (Kennedy and Purves, 1941) a positive goitrogenic agent was shown to be of general occurrence in the seeds of Brassica species. The thyroid stimulating effect of this agent was apparently unrelated to iodine deficiency, and manifested itself even in rats which received 1 mg. of iodine daily, injected in the form of iodide. MacKenzie and MacKenzie (1943), and Astwood, Sullivan, Bissell and Tyslowitz (1943) have recently reported the goitrogenic action of sulphonamide derivatives in the rat, and have found that the action of these substances on the thyroid was likewise uninhibited by large doses of iodine. The action was, however, abolished by the administration of thyroid substance or thyroxine. This paper reports the effects of injections of di-iodotyrosine and of thyroxine on the goitrogenic action of Brassica seeds.

Methods.

Male rats between 80 and 100 g. were placed on the rape seed diet as previously described (Kennedy and Purves, 1941). Concurrently with the administration of the diet they were given daily injections of the substance whose effect was to be tested. The dosage was:

Iodide: 1 ml. per day of 0·13 per cent. solution of potassium iodide in saline.

Di-iodotyrosine: 1 ml. per day of 0·17 per cent. solution in saline.

Thyroxine: 0·25 ml. per day of a solution containing 4·0 mg. r-thyroxine and 40 mg. of sodium carbonate in 100 ml. of saline.

The dosages of iodide and di-iodotyrosine were so chosen in order that each should contain 1 mg. of iodine.

In all cases there were six rats for each test and the duration of the experiments was 21 days.

In subsequent experiments with thyroxine injections a solution containing 1·0 mg. thyroxine per 100 ml. was used. The rats were weighed each day and the dosage calculated from the body-weight.

Results.

Table I shows the results of iodide, di-iodotyrosine, and thyroxine injections on the weight and histology of the thyroids. The iodide and the di-iodotyrosine injections produced equivalent effects, both reducing the weight response and modifying but not preventing the hyperplasia. The thyroxine injections completely inhibited the thyroid hyperplasia usually induced by the rape seed diet.

Table II shows the results of tests to determine the minimum amount of thyroxine necessary to inhibit the action of the rape seed diet.

The results show that between 2 and 3 μg. of thyroxine per 100 g. body-weight per day is necessary to inhibit the action of the rape seed diet. Experiments with daily doses of 2·5 μg. thyroxine per 100 g. body-weight have yielded varying results, sometimes inhibiting the action and sometimes failing.

TABLE I.—*Rats on Rape Seed Diet.*

Injections.	Average body-weight.	Average thyroid weight.	Thyroid histology.
Nil	129 g.	41·8 mg.	Intense hyperplasia.
1·3 mg. pot. iodide per day	124 g.	19·0 mg.	Moderate ,,
1·7 mg. di-iodotyrosine	124 g.	20·0 mg.	,, ,,
10 μg. r-thyroxine per day	120 g.	10·8 mg.	Complete inactivity.
Control on stock diet	126 g.	12·6 mg.	Slight " normal " activity.

TABLE II.—*Rats on Rape Seed Diet injected with Thyroxine.*

Dose of thyroxine per 100 g. body-weight per day.	Thyroid : average weight per 100 g.	Thyroid histology.
10 μg.	9·3 mg.	Complete inactivity.
9 ,,	8·8 ,,	,, ,,
8 ,,	9·3 ,,	,, ,,
7 ,,	9·1 ,,	,, ,,
6 ,,	8·8 ,,	,, ,,
5 ,,	8·5 ,,	,, ,,
4 ,,	8·3 ,,	,, ,,
3 ,,	8·0 ,,	,, ,,
2 ,,	19·0 ,,	Moderate hyperplasia.
1 ,,	20·3 ,,	Marked ,,
0·5 ,,	24·9 ,,	,, ,,
Nil	32·2 ,,	,, ,,

DISCUSSION.

The most striking feature is the abruptness with which the hyperplasia disappears as the thyroxine dosage is increased above 2·0 μg. per 100 g. body-weight. This effect promises to be useful as a method of assay of preparations with thyroxine activity.

Since it has been shown that the thyroid hyperplasia induced by rape seed diet is due to excessive secretion of thyrotropin, the action of thyroxine in inhibiting this hyperplasia is to be ascribed to an inhibition of the thyrotropin secretion by the pituitary.

MacKenzie and MacKenzie (1943) and Astwood, Sullivan, Bissel and Tyslowitz

(1943) consider that the action of the sulphonamides and thioureas on the thyroid is primarily an impairment of thyroxine synthesis, a conclusion that had already been reached by us for the mechanism of the action of the Brassica seed agent. It is considered that the seeds contain a substance which, after absorption, acts on the thyroid and inhibits or interferes with its power to synthesize thyroxine. The resultant fall in the level of thyroxine in the circulation induces histological changes in the pituitary identical with those produced by thyroidectomy. Functionally the pituitary responds by an outpouring of thyrotropin, which acts on the thyroid and produces the histological activation which is observed. The hyperplasia of the thyroid is thus an attempt by the pituitary-thyroid team to maintain the normal output of thyroxine synthesis. This hypothesis explains all the facts at present known about the effects of positive goitrogenic agents. It is supported by evidence which shows that 2·5 μg. of r-thyroxine is approximately equivalent to the normal daily requirement of thyroid hormone in a 100 g. rat. Thus Hohlweg and Jenkmann (1933) determined the amount of synthetic thyroxine necessary to prevent thyroidectomy changes in the pituitary of the thyroidectomized rat, and found the requirement of a 50–60 g. rat to lie between 1·0 and 2·5 μg. Evans, Simpson and Penchartz (1939) obtained normal growth and metabolism in thyroidectomized rats up to 200 g. body-weight by the administration of 5·0 μg. of thyroxine daily. Rowlands (1942) obtained normal growth in thyroidectomized rats of about 100 g. body-weight by the administration of 2·5 μg. of thyroxine daily.

The abruptness with which thyroid hyperplasia appears when the amount of thyroxine injected is reduced below 2·5 μg. daily is also consistent with the view that the functional efficiency of the thyroid tissue is greatly impaired. Thus when 2·0 μg. of r-thyroxine is injected daily the pituitary-thyroid team is called on for the supply of the equivalent of only 0·5 μg. of r-thyroxine daily, but even this small demand evokes a considerable degree of thyroid hyperplasia.

The effect of di-iodotyrosine in moderating the thyroid hyperplasia produced by rape seed diet is the same as that of an equivalent amount of iodide. This evidence suggests that the goitrogenic agent interferes with the later stages of thyroxine synthesis in the thyroid, i.e. with the conversion of di-iodotyrosine to thyroxine. This evidence is, however, inconclusive, since it has not been shown that the normal thyroid is able to utilize injected di-iodotyrosine directly.

SUMMARY.

1. r-thyroxine in daily doses of 3·0 μg. per 100 g. body-weight abolishes the thyroid hyperplasia induced by feeding rats on rape seed diet.

2. Iodide and di-iodotyrosine are equally effective on the basis of iodine content in moderating the thyroid activating effect of the rape seed diet. They do not abolish the action even in large doses.

3. The action of the positive goitrogenic agents can be explained by the hypothesis that they interfere with the synthesis of thyroxine by the thyroid cells.

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STUDIES ON EXPERIMENTAL GOITRE. V. PITUITARY FUNCTION IN RELATION TO GOITROGENESIS AND THYROIDECTOMY.

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RECENT work on experimental goitre in rats (Griesbach, 1941) has shown that thyroid hyperplasia, produced by a goitrogenic substance present in numerous seeds, is always accompanied by conspicuous cell changes in the pituitary gland. It has further been shown (Griesbach, Kennedy and Purves, 1941) that thyroid hyperplasia cannot be produced or maintained in the absence of the pituitary.

From these results it is clear that the active principle of Brassicae seeds cannot stimulate the thyroid directly, but requires the mediation of the thyrotropic hormone. These experiments did not determine whether the sensitivity of the thyroid to physiological amounts of thyrotropin is increased by the goitrogenic factor, or the pituitary stimulated to secrete abnormal amounts of this hormone. In Section I of this paper direct evidence is produced that the latter conception is correct. An increased thyrotropic activity in the serum of rats with experimental goitre has been demonstrated, while the sensitivity of the rat's thyroid to injected thyrotropin has been found not to be increased by the goitrogenic agent.

The occurrence of marked histological changes in such pituitaries which obviously secreted more thyrotropin suggested that measurements of the hormone content of these glands might, by correlation with the level of cell types, lead to some deductions concerning the site of production of the thyrotropic hormone. Experiments dealing with this question are presented in Section II. The problem encountered is similar to the one concerning the interpretation of the "thyroidectomy" changes in the pituitary. In spite of recent investigations (Zeckwer, 1935, 1936; Turner and Cupps, 1940) the question as to the amount of thyrotropin present and its relation to the pituitary cell changes after thyroidectomy cannot be considered as settled. Since the "thyroidectomy" changes are probably closely related to the "goitre" changes, "thyroidectomy" pituitaries have been included in the present assay experiments.

I. THE THYROTROPIN CONTENT OF THE SERUM AND THE THYROTROPIN SENSITIVITY OF RATS ON BRASSICA SEED DIET.

Materials and Methods.

The rats used were of a Wistar strain and were about four months old at the time of assay. Three diets were used, as follows:

Diet.	S1.	S2.	G1.
Bran	30	30	..
Pollard	25	25	..
Bone meal	15	15	..
Meat meal	15	..	..
Skim milk powder	..	15	30
Maize meal	15	15	25
Ground rape seed	..	..	45
	100	100	100

In addition animals on diets S1 and S2 received whole barley and kibbled maize *ad lib.*

Blood was collected by aspiration from the heart of rats under ether anaesthesia. The separated serum was treated by the addition of 0·3 per cent. tricresol and kept in the ice chest until used. The serum was not stored longer than one week before use. In this way serum was obtained from three groups of rats : normal rats on diet S1, thyroidectomized rats on diet S1, and rats on the goitrogenic diet G1.

For the test of thyrotropic activity the recipient guinea-pigs had to be pretreated for two reasons. Firstly, it was necessary to exclude the possibility that the stimulation of the guinea-pig thyroid might be due to the goitrogenic factor of the diet carried over in the serum of the rat. Secondly, in order that the small degree of stimulation expected might be significant, it was necessary that guinea-pig thyroids should be uniform in histological activity. Both these ends were achieved by injecting the guinea-pigs from birth until the end of the experiments with 10 μg. of r-thyroxine per day. This thyroxine treatment was found to inhibit the action of goitrogenic agents on the guinea-pig thyroid as it does in the rat (Purves, 1943). Furthermore, we have shown (Griesbach and Purves, 1943) that these doses of thyroxine reduce the variation in the cell height measurements of the guinea-pig thyroid and increase its sensitivity to injected thyrotropin. We feel justified to assume that by this procedure we have produced a test animal with a specificity of response approaching that of the hypophysectomized animal by physiologically inhibiting the thyrotropic activity of the animal's own pituitary.

The sera to be tested were injected into the guinea-pigs, beginning when they were three weeks old. The sera tested were given in a total dosage of 10 ml. per 100 g. body-weight, two injections of 1·25 ml. per 100 g. intraperitoneally being given on each of four successive days. On the fifth day the animals were sacrificed, the thyroid dissected out, fixed in formol saline and sectioned. Sections were stained by Heidenhain's azan, and projected on to a screen at a magnification of 1000. The mean acinar epithelium height (hereafter called cell height) was obtained by measuring 100 cells in each gland with a celluloid scale by the method described in the preceding paper.

For the thyroid sensitivity test two groups of rats were used. One received rape seed diet G1, the other stock diet S1. Both were injected subcutaneously with 10 μmg. thyroxine per day until the end of the experiment. By this means the hyperplasia usually induced by the rape seed diet was prevented and the thyroids brought to a resting state. After three weeks graded doses of thyrotropic hormone preparation (Flavianate, after Evans, Meyer and Simpson, 1933) were injected for five days. The rats were sacrificed on the day after the last injection, and the thyroids dissected out. The histological technique and cell height measurements were carried out in the same way as for the guinea-pig thyroids.

Results.

(a) *Serum thyrotropin.*

The results of the serum assays are shown in Table I.

TABLE I.—*Thyrotropin Assay of Rat Sera.*

Serum.	Number of guinea-pigs.	Mean cell height of thyroids.	Increase over control.
Nil (control)	10	3·14μ	—
Normal rat	4	4·04μ	0·90μ
Thyroidectomized rat	4	5·88μ	2·74μ
Rape seed diet 14 days	3	5·68μ	2·54μ
,, ,, 28 days	1	5·96μ	2·82μ

t Test of Significance.

Comparison.	Difference.	P.
Normal—control	0·90μ	<0·02
Thyroidectomy—normal	1·84μ	<0·01
Rape seed 14 days—normal	1·64μ	<0·02
,, 28 ,, — ,,	1·92μ	<0·02
Thyroidectomy—rape seed 14 days	0·10μ	0·8 not significant.

As was expected the effects were weak, but activity was demonstrated even in the normal rats' serum. Both the thyroidectomized rats' serum and that from rape seed

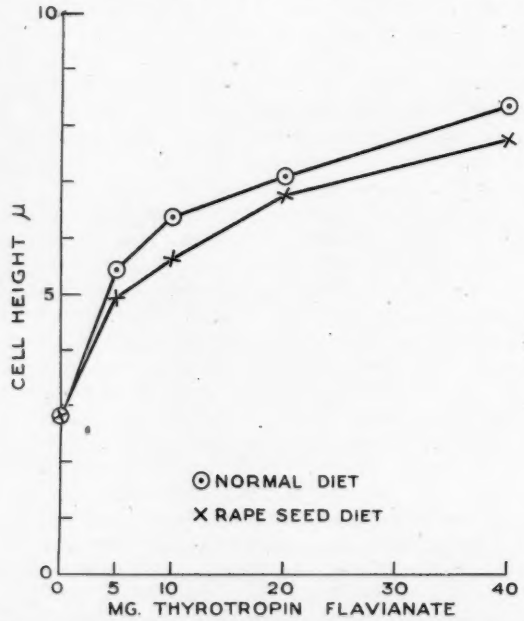


FIG. 1.—Response of rat thyroids to thyrotropin injections.

fed rats were significantly enriched in thyrotropic activity as compared with normal rats' serum.

(b) *Thyroid sensitivity.*

The results of the tests of sensitivity of rat thyroids to thyrotropic hormone injections are given in Table II, and are shown graphically in Fig. 1.

TABLE II.—*Response of Rat Thyroids to Thyrotropin Injections.*

Dose of hormone.	Cell heights of thyroid.	
	Normal diet.	Rape seed diet.
0	3·0μ	2·9μ
5 mg.	5·6μ	5·0μ
10 ,,	6·6μ	5·7μ
20 ,,	7·1μ	6·8μ
40 ,,	8·4μ	7·7μ

The cell heights of the thyroids of rats on rape seed diet were at all thyrotropin doses slightly below the values for the rats on normal diet, though this difference is not significant. The results show that the sensitivity of the rat thyroid is not increased by the action of the goitrogenic principle of rape seed.

II. THE THYROTROPIN CONTENT OF THE PITUITARY IN EXPERIMENTAL GOITRE AND AFTER THYROIDECTOMY.

Materials and Methods.

The pituitary thyrotropic hormone assays were performed on 3–4 weeks old guinea-pigs of both sexes, weighing 180–240 g. In experiments III, IV and V the guinea-pigs were pretreated with iodide, as described in our previous paper (Griesbach and Purves, 1943). Eight guinea-pigs were used for each assay. Fresh rat pituitaries were obtained each day, weighed, ground up in an agate mortar and suspended in sterile saline. The guinea-pigs were injected intraperitoneally with 1 ml. of pituitary suspension, equivalent to one half of a rat's pituitary, for five consecutive days. A total of 20 rats were therefore used for each assayed group. Four additional pituitaries from each group were obtained for histological examination. The histological methods were as previously described (Griesbach, 1941). Evaluation of the assay results was done both from the weight of the guinea-pig thyroids and from the mean acinar cell heights. The technical details are described in the previous paper (Griesbach and Purves, 1943). As expected, the thyroid weights proved to be less sensitive and a less accurate measure of thyrotropic activity. Within these limitations the thyroid weight response paralleled the cell height response, and there was no evidence that these whole pituitary extracts behave differently from the flavianate preparation used for the standardizing curves in the previous paper. Using the regression equations given there, the relative thyrotropic contents were calculated from the mean cell heights.

Differential cell counts of pituitary sections were made, using a ruled cover-slip in the eyepiece of the microscope to divide the field. The area counted consisted of a strip parallel to the long diameter of the section, excluding the marginal zone. The numbers counted in each gland always exceeded 1200 cells.

The effect of thyroidectomy on the pituitary thyrotropin was not only examined by the guinea-pig method, but in addition, following the suggestion of A. Severinghaus

(1937), a few assays were performed with hypophysectomized rats as test animals. Each rat, after showing a fairly constant weight curve, 2–3 weeks following hypophysectomy, received one pituitary twice daily for 3 days, by intraperitoneal grafting through a wide needle. The rats were killed 48 hours after the last injection and the thyroids examined.

Results.

Table III summarizes the results, obtained in five separate experiments, each comprising a series of assays carried out simultaneously. Owing to variations in animal respose, etc., comparison between the assays of one experiment may be more valid than comparison of the results of one experiment with another.

TABLE III.

Diet of rat.	Average weight of pituitary. (Mg.)	Thyrotropin units.* Per pituitary.	Thyrotropin units.* Per 10 mg. wet weight.	Histology of pituitary. Acidophils. (%.)	Basophils. (%.)	Chromophobes. (%.)	Number of cells counted.
Experiment I.							
A. Normal : S1	6·75	7·6	11·0	..	..	..	..
B. Rape seed : 21 days	5·41	0·9	1·6	29·1	27·3	43·6	2,669
C. „ : 21 „ plus normal S1 8 days	6·08	6·0	9·9	46·9	15·4	37·7	2,868
Experiment II.							
A. Normal : S2	5·81	8·4	15·4	43·8	6·5	49·7	5,572
B. Rape seed : 21 days	4·10	9·8	23·9	41·5	12·1	46·4	12,081
C. Thyroidectomy : S2	8·73	2·7	3·1	3·5	24·9	71·6	3,882
D. „ : Rape seed	6·70	1·7	2·5	2·3	31·7	66·0	7,235
Experiment III.							
A. Normal : S1	4·00	6·3	15·8	46·9	4·8	48·3	11,717
B. Rape seed : 28 days	4·41	5·4	12·3	42·3	28·6	29·1	4,767
Experiment IV.							
A. Normal : S1	5·04	8·2	16·2	46·9	4·8	48·3	11,717
B. Rape seed : 14 days	4·48	0·9	2·0	39·1	26·9	34·0	10,760
C. Thyroidectomy : S1	9·75	10·1	10·3	43·7	28·2	28·1	6,342
D. „ : Rape seed	8·11	1·1	1·4	1·2	33·5	65·3	8,375
Experiment V.							
A. Normal : S1—Young rats	5·78	9·0	15·6	46·9	4·8	48·3	11,717
Adult rats	11·18	5·8	4·9	46·3	4·2	49·5	7,500
B. Thyroidectomy : S1	8·49	6·9	8·2	38·2	29·4	32·4	4,257

* Calculated from the equations : Experiments I and II : Log. U = 0·236 C.H. (μ) − Log. Wt. G–P (g.) −4·01

Experiments III, IV and V : „ = 0·198 „ − „ „ „ −3·29

(a) *Normal rats' pituitaries.*

Experiments II, III, IV and V, which were done with rats about 10 weeks old, gave results in close agreement with an average of 15·8 units of thyrotropin per 10 mg. pituitary. Adult rats (Expt. V), one year old, gave a much lower value—4·9 units per 10 mg. In Expt. I, rats 4 months old gave an intermediate value—10·9 units per 10 mg. It is evident that the thyrotropin concentration in the pituitary diminishes considerably with age. This corresponds to the results of Turner and Cupps (1939), who found increased concentration during the period of rapid growth, followed by a steep fall after reaching maturity. The difference in the thyrotropin content between the normal male and female pituitary, as described by Turner and Cupps, could not be found in our experiments on immature rats.

There is no significant change in the differential cell count corresponding to the age changes in hormone concentration, but the amount of acidophil substance per acidophil cells seems to be lower in the older animals (Wolfe, Bryan and Wright, 1938). It is quite possible that there is a close correlation between the amount of acidophil *substance* and thyrotropic *concentration*, but this is not revealed by the results of the differential count, which takes no account of the grade of granulation in the acidophils.

(b) *Effect of thyroidectomy.*

The result was found to depend on the diet which the rats received. On stock diet S2 thyroidectomy regularly leads to almost complete degranulation of the acidophils. On stock diet S1, which contains commercial meat meal, the acidophils are always retained in relatively normal condition. This is probably the cause of the difference in gross appearance of the two types of rat pituitaries. The anterior lobes, after complete thyroidectomy, containing no acidophil granulation, are transparent, almost gelatinous, while the others are opaque, like the normal. We have found this a valuable macroscopic sign, from which we have found no exception. This difference in relation to the acidophil cells we have reason to ascribe to small amounts of thyroxine in the meat meal. Experimental evidence has shown that these effects could be produced by administration of between 0·5 and 2·5 μg. of r-thyroxine per 100 g. rat per day. Thyroidectomized rats on rape seed diet gave results similar to those on stock diet S2—that is, the acidophil granulation was almost completely lost. The characteristic basophil change was not visibly affected by the diet, approximately 30 per cent. of the cells being of this type. Greater amounts of thyroxine are apparently necessary to cause reversal of the basophil transformation. Owing to the large size and extensive vacuolation of these basophils they comprise much more of the bulk of the pituitary tissue than their numbers would indicate.

The thyrotropin concentration also depends on the diet. On stock diet S2 and on the rape seed diet thyroidectomy produced very low levels of thyrotropin concentration. The lowest level was found in thyroidectomy rape seed diet in Expt. IV, where the thyrotropin concentration, 1·4 units/10 mg. was only 8·7 per cent. of the normal level. Owing to the greater weights of thyroidectomy pituitaries the absolute thyrotropin contents are not reduced in the same proportion, but are still much less than normal.

On stock diet S1 (meat meal) only a small decrease in thyrotropin was produced by thyroidectomy. The average thyrotropin concentration from Expts. IV and V was 9·2 units/10 mg., which represents 59 per cent. of the normal value. The increased

TABLE IV.—*Pituitary Assay on Hypophysectomized Rats.*

Donor rats.				Thyroid response in hypophysectomized rats.	
Diet treatment.	Number and sex of rats used.	Mean weight. (g).	Pituitary weight injected. (mg.)	Histology.	Cell height. (μ.)	Increase. (μ.)
Normal	3 ♂ 3 ♀	125	37·1	Normal	7·1	4·5
„	3 ♂ 3 ♀	125	38·7	„	6·9	4·3
Thyroidectomy: 60 days Normal diet: S1	3 ♂ 3 ♀	147	50·5	Slight degranulation of acidophils, 25 per cent. basophils	4·8	2·2
Thyroidectomy: 60 days Rape seed: 34 days	3 ♂ 3 ♀	103	50·5	Extensive degranulation of acidophils, 26 per cent. basophils	3·9	1·3
					Control 2·6	—

size of the pituitaries compensated for this decreased concentration, the absolute thyrotropin content per pituitary being in Expt. IV above normal, in Expt. V somewhat below, and the average value not significantly different from the normal value for rats of this age.

Four experiments, reported in Table IV, were carried out on hypophysectomized rats. Corresponding to the low sensitivity of the rat thyroid no significant weight increases were produced by pituitary implants. The results of cell height measurements were, however, unequivocal. The thyroidectomy pituitaries contained distinctly less thyrotropic hormone, per gland, than the normal pituitary. This fact is even more marked if the potency per weight unit of pituitary is considered. These experiments, originally started on the ground of a critical remark of A. Severinghaus (1937) that thyroidectomy pituitaries should be assayed on hypophysectomized animals, were not done on a large scale, but the fact that the results correspond to those obtained by the guinea-pig method suggests that for the problems under investigation the assay on the not hypophysectomized guinea-pig is equally reliable.

(c) *Effect of goitrogenic diet.*

The effect of the diet was not consistent. In Expts. II and III the characteristic basophil changes were present in the pituitaries, but the acidophil percentage and the amount of granules within the cells were only slightly decreased. In both cases the absolute thyrotropin content was not significantly different from normal. Owing to the pituitary weight in Expt. II being less for the rape seed diet rats than for their normal controls, the calculated thyrotropin concentration is for this group greater than normal. The growth of these rats was retarded and this probably accounts for the lower pituitary weight.

In Expts. I and IV the degranulation was more advanced, and most of the remaining acidophils were reduced to a mere rim or crescent of acidophil granules round the nucleus. The reduction of acidophil substance is thus much more extensive than the percentage figure would indicate. In both these experiments low values were obtained for pituitary thyrotropin content, both absolute and for the concentration of the hormone. The combined results give a thyrotropin concentration of about 14 per cent. of the normal.

An additional group in Expt. I, after 3 weeks on rape seed diet, was transferred for eight days to stock diet S1. In these animals the thyrotropin concentration was found to be restored to normal. The acidophils were regranulated and were present in normal proportions. On the other hand, the basophils remained in excessive numbers. Their appearance, however, was altered, the hyaline accumulations had been lost and the cytoplasm was filled with coarse basophil granules, resembling the basophil appearances produced early after castration.

DISCUSSION.

The results of the thyrotropin assays on the sera are unequivocal. In the rats with thyroid hyperplasia produced by the rape seed diet the serum has a much greater thyrotropic activity than that of normal rat serum. The thyrotropic effect of the serum of the rape seed diet rats was indeed equivalent to that of thyroidectomized rats, and this is in accordance with the histological appearance of the thyroids of rape seed fed rats, which is one of maximum activity. Cope (1938) found in the sera of rabbits after thyroidectomy a significant thyrotropic activity which was usually absent in the sera of normal rabbits. In our experiments with pooled rat serum, the normal serum showed a small but significant activity. The response in the guinea-pig thyroids was not large enough to bring the cell height on to the straight line portion of the response

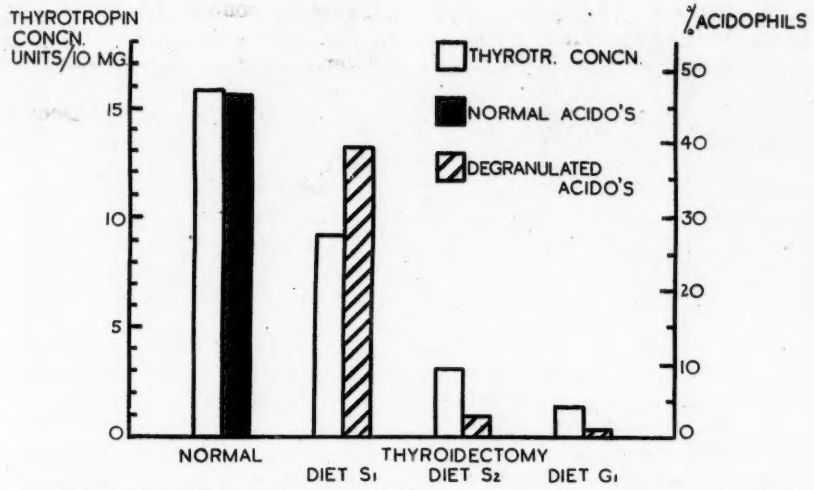


FIG. 2*a*.—Effect of thyroidectomy on pituitary thyrotropin and pituitary acidophil cells. Degranulation of acidophils was more extensive than the thyrotropin depletion.

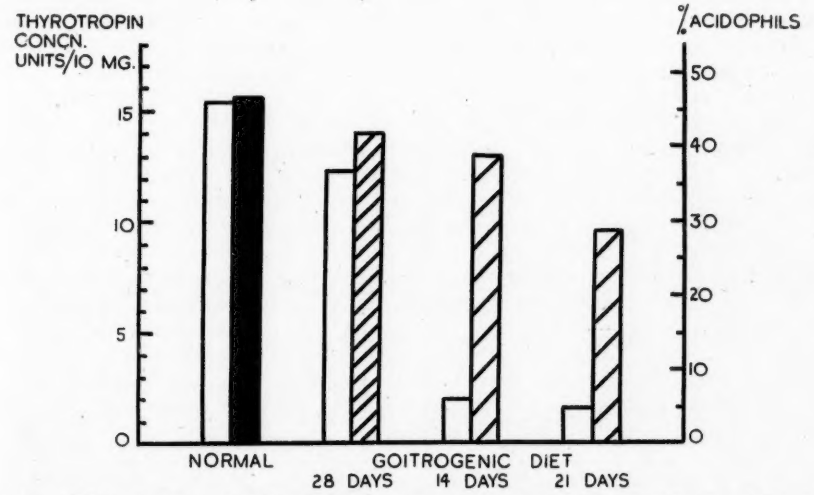


FIG. 2*b*.—Effect of goitrogenic diet on pituitary thyrotropin and pituitary acidophil cells. Depletion of thyrotropin was more extensive than the degranulation of the acidophils.

curve. We cannot, therefore, say what the relative thyrotropin contents of goitre and thyroidectomy sera are, compared with normal serum, although the former are plainly much greater. The sensitivity of the rat thyroid to thyrotropin is not increased by the rape seed factor. The thyroid hyperplasia which appears in rats on the rape seed diet is entirely due to increased thyrotropin secretion by the pituitary.

Severinghaus (1937) has pointed out that there may not be a direct relation between the hormone content of a pituitary and its rate of secretion. Rapid secretion may be

accompanied by a high hormone content, as is true for the gonadotropic secretion, or may be accompanied by low hormone content, the result of depletion. The results of our pituitary assays show that in the states where rapid thyrotropin secretion obtains, i.e. thyroidectomy and intense thyroid hyperplasia, the thyrotropin content of the pituitary is depleted. The extent of this depletion varies. Thyroidectomized rats on diet S1, which contained meat meal showed only a small reduction in thyrotropin concentration (see Expts. IVc and Vb, Table III). Thyroidectomy on diet S2 or on the rape seed diet showed a great reduction in thyrotropin concentration. This depletion effect is therefore dependent on the removal of the last traces of thyroxin from the animal. If the effects of the rape seed factor are to be ascribed to the interference with the thyroxine synthesis in the thyroid, then the depletion of pituitary thyrotropin in Expts. Ib and IVb would indicate that in these animals the thyroxine secretion of the thyroid was almost completely inhibited.

An examination of the correlation between the pituitary thyrotropin concentration and the histology shows that thyrotropin depletion is accompanied by degranulation of the acidophil cells. The relevant data are summarized in the diagram in Fig. 2. The parallelism between the thyrotropin concentration and the acidophil percentage which is apparent in this diagram led us to consider the possibility that the acidophils might be responsible for the production of thyrotropic hormone, as suggested by Marine, Rosen and Spark (1935), and that the acidophil granules might represent, in part at least, stored thyrotropic hormone. A quantitative comparison between the observed thyrotropin concentration and the amount of acidophil substance present in these pituitaries is made difficult by the fact that, firstly, the acidophils are degranulated to a varying extent, and secondly, the basophils are so large individually that they occupy more space than their relative numbers indicate. The percentage count of acidophils is therefore not by any means proportional to the amount of acidophil substance present in the unit volume of pituitary. We had therefore hoped to overcome this difficulty by obtaining for assay putuitaries practically free of acidophil substance. Thyroidectomy rape seed diet (Expt. IVd) was the nearest approach to this condition. The 1·2 per cent. acidophils recorded were so scantily equipped with acidophil granules that the actual amount of acidophil substance was clearly much less than 1/100 of the normal value. The thyrotropin concentration found, 8 per cent. of the normal, is far from being proportional to this value. However, this amount of thyrotropin could have come from one or two incompletely thyroidectomized rats' pituitaries in the 20 used for the assay—a possibility we are not able to exclude with certainty, since at that time we could not yet recognize completeness of thyroid ablation by the gross appearance of the pituitary (see above). The smallest remaining fragment of thyroid leads to retention of acidophils which could have provided the amount of hormone found.

DESCRIPTION OF PLATE.

Fig. 3.—Thyroid of control guinea-pig. Cell height 3·14μ. Azan, × 470.

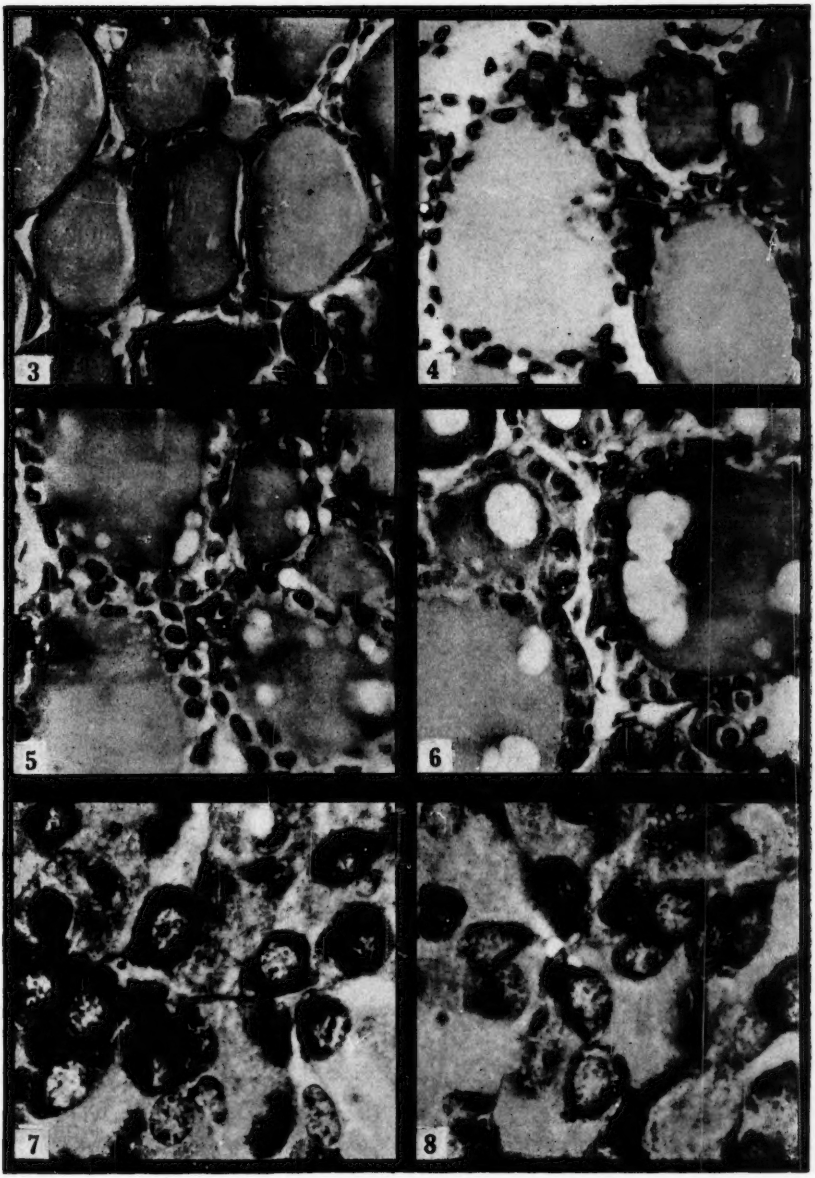
Fig. 4.—Thyroid of guinea-pig injected with normal rats' serum. Cell height 4·04μ. Azan, × 470.

Fig. 5.—Thyroid of guinea-pig injected with thyroidectomized rats' serum. Cell height 5·88μ. Azan, × 470.

Fig. 6.—Thyroid of guinea-pig injected with serum from rats on rape seed diet. Cell height 5·68μ. Azan, × 470.

Fig. 7.—Pituitary of rat 28 days on rape seed diet. (Exp. IIIb.) The acidophil cells are well filled with granules. There was no significant depletion of pituitary thyrotropin in this experiment. Mallory, × 1100.

Fig. 8.—Pituitary of rat 14 days on rape seed diet. (Exp. IVb.) The acidophil cells show extensive degranulation. The remaining granules are concentrated at the cell periphery forming rings or crescents. There was extensive depletion of pituitary thyrotropin in this experiment. Mallory, × 1100.



Griesbach and Purves.

Contrary to the situation following thyroidectomy, there are obvious discrepancies between the hormone concentration and the acidophil counts of rape seed diet pituitaries which even the varying degrees of degranulation, though certainly present, would be insufficient to explain. The lack of parallelism between the thyrotropin concentration and the amounts of acidophil substance does not exclude the possibility that the thyrotropic activity resides in these granules. The granules may not be all of the same kind, or they may not have a constant thyrotropic concentration.

In a previous publication (1941) we suggested that the signs of increased activity in the basophil cells of rape seed diet pituitaries pointed to the possibility that this type of cell was responsible for the secretion of thyrotropic hormone. A re-examination of this assumption is now possible, using the relative values of serum thyrotropin, given in Section I as an indication of the rate at which thyrotropin is being secreted.

TABLE V.—*Pituitary Cell Types in Relation to Thyrotropin Concentration in Pituitary and Serum.*

State of rats.	Pituitary thyrotropin. (Units/10 mg.)	Pituitary cells.		Serum thyrotropin cell height response. (μ.)
		Acidophils. (%.)	Basophils. (%.)	
Normal	15·8	46·9	4·8	0·90
Rape seed diet, Expt. IIIB	12·3	42·3	28·6	2·82
,, ,, ,, IVB	2·0	39·1	26·9	2·54
Thyroidectomy, ,, IVD	1·4	1·2	33·5	2·88

The arrangement from above down is considered to represent increasing severity of thyroxine deprivation. The depletion of pituitary thyrotropin and pituitary acidophil cells increases from above downwards. The increase in the thyrotropic effect of the serum runs parallel to the increase in pituitary basophil cells.

The data are summarized in Table V, which shows the data for those groups of animals from which both serum and pituitary thyrotropin values were obtained. In the right half of Table V it is seen that there is a close correlation between the thyrotropic activity of the serum and the percentage of basophils in the pituitary. This evidence for the basophil as the site of thyrotropin *production* is quite as convincing as that given above for the acidophil as the site of *storage* of thyrotropin. This fact only shows how much caution must be used in trying to ascribe the production of a pituitary hormone to one of the observed cell types. To link a hormone to a particular cell type it would be necessary to show that changes in the activity or numbers of only *one* type of cell are accompanied by changes in the content or secretion of only *one* hormone. Here we have, on the one hand, at least two marked cell disturbances and, on the other, no evidence as to how the pituitary hormones other than thyrotropin may be affected. One thing is clear—the hyaline material which accumulates in the basophils does not contain thyrotropin, as the latter is at its lowest level when the hyaline is most abundant. Furthermore, assay Ic, in which pituitaries containing three times the normal number of coarsely granulated basophils showed only normal thyrotropin concentration, indicates that the thyrotropin is not in the basophil granules. If it had been in these granules we would have expected values of thyrotropin concentration much greater than normal. If the thyrotropin is present in the pituitary in histologically visible form, it would have to be present in the acidophil granules, but it is, *a priori*, as likely that the thyrotropin does not exist in any cytological detail, demonstrable by present technique.

SUMMARY.

1. The thyrotropin concentration in serum of rats fed on Brassica seed diets is raised to the same level as that induced by thyroidectomy. The thyroid hyperplasia produced by such diets is entirely due to the increased secretion of thyrotropin by the pituitary.

2. Thyroidectomy in rats maintained on a diet not containing meat meal causes a great reduction in the thyrotropin concentration of the pituitary, accompanied by almost complete disappearance of acidophil substance.

3. Thyroidectomy in rats maintained on a diet containing meat meal causes only moderate reduction of pituitary thyrotropin and acidophil substance. Such pituitaries are similar in appearance to those produced by subtotal thyroidectomy, or by the administration of small amounts of thyroxine to completely thyroidectomized rats on a meat-free diet.

4. Goitrogenesis by the rape seed diet was accompanied by varying degrees of reduction of pituitary thyrotropin and acidophil substance. The analogy between the effects of goitrogenic diet and of thyroid ablation supports the view that the thyroid hyperplasia produced by the goitrogenic diet is secondary to impairment of thyroxine synthesis.

5. A quantitative investigation has been made of the four changes produced by thyroid disturbances, viz. increase of serum thyrotropin, depletion of pituitary thyrotropin, increase in number and activity of pituitary basophil cells, and degranulation of pituitary acidophil cells. The correlations observed are to be ascribed to the operation of a common cause—thyroxine deficiency—and cannot be used to locate the site of production of thyrotropic hormone.

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THE ASSAY OF THYROTROPIC ACTIVITY BY THE CELL HEIGHT RESPONSE IN GUINEA-PIGS.

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IN investigations on the goitrogenic action of Brassica seed diets the authors found it necessary to assay rat pituitaries for thyrotropic activity. The sensitive test animals available for this assay are the chick and the guinea-pig. Our experience with the chick thyroid weight method of Smelser (1938) was disappointing. Salter's (1940) method of microhistometric evaluation of the chick thyroid response was also unsuitable in this country, as the one-day-old chick has already here a hyperplastic thyroid. We therefore turned to the guinea-pig as a test animal.

The guinea-pig thyroid weight method (Rowland and Parkes, 1934; Bergman and Turner, 1939) was more successful. We found, however, the thyroid weights of our guinea-pigs were so variable that to attain the accuracy we required would have necessitated a prohibitive expenditure of animals. The use of the microhistometric technique to evaluate the response of the guinea-pig thyroid (Rawson and Starr, 1938) was found to provide the necessary sensitivity and accuracy while retaining the advantages of an objective measurement. However, having regard to the time consumed by the tedious measurements of large numbers of cells, one must agree with Lambie (1939) that the method does not commend itself from the practical standpoint in its original form. We present here a method by which the necessary measurements of cell height can be more readily obtained than by the eyepiece micrometer method of Starr. Moreover we shall show that it is unnecessary to measure such large numbers of acini as have been considered necessary (100 or more per thyroid).

MATERIAL AND METHODS.

Projector.

An apparatus was constructed to enable projection of the thyroid sections on a white screen at a magnification of 1000. The illumination is provided by a 6–8 volt 32 watt automobile headlight globe. The current is supplied by a transformer. The condenser is one made for a small photographic enlarger, and is arranged to throw an image of the filament on to the back lens of the microscope condenser.

The microscope is clamped into position. The position of the screen is calculated from the published magnifications of the high-power dry objective ($\times$ 45) and eyepiece ($\times$ 10) used, and checked by projecting the image of a stage micrometer so that the magnification is exactly 1000.

The screen is covered with several coats of white enamel undercoat paint and rubbed down to a smooth matt surface with fine sandpaper.

For the measurement, use is made of a celluloid scale shown in Fig. 1. This consists of two lines diverging with a taper of 1 in 10. Each line is marked and numbered in centimetres; the numbers therefore indicate millimetres' distance between the lines at each position.

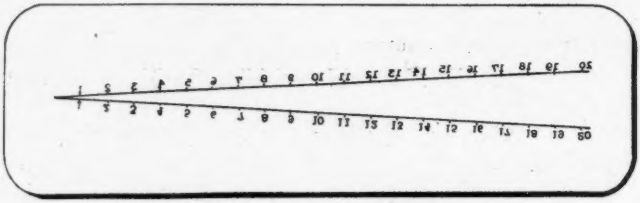

FIG. 1.—Celluloid scale for cell height measurements (reduced).

The ink used is compounded as follows:

Crystal violet	1·0 g.
Shellac	6·0 g.
Glacial acetic	100 g.

Dissolve by boiling.

The numbering should be done in reverse, so that in use the markings are against the screen.

Animals.

The guinea-pigs used were killed at about 4 weeks of age, when their weights were between 180 and 230 g. Those animals which received thyrotropin injections were injected for five consecutive days, beginning at about 23 days of age, and were killed on the day after the last injection. Iodide treatment, when given, was begun on the third day from birth, and 0·2 mg. potassium iodide in saline injected every second day until five injections were given. When thyroxine treatment was given, 10μg. of r-thyroxine in alkaline solution were injected daily, beginning on the third day from birth and continuing until the animals were killed.

Histological technique.

The guinea-pigs were killed with coal-gas and thyroids dissected out, weighed, and fixed in 10 per cent. formol saline. They were dehydrated in alcohol, cleared in cedar wood oil, and embedded in paraffin so that the long dimension of the gland was parallel to the surface of the block. Sections were cut at 5μ. It is important that the sectioning be carried well into the centre of the thyroid, as a tangential section does not give a representative sample. The sections were stained by Heidenhain's azan method, which gives a good definition of the cell borders, and is much superior to haematoxylin-eosin staining for cell height measurements.

Measurement.

The slide was placed in the projector in a darkened room and measurements were made up and down a strip parallel to the long diameter of the section. Well defined acini were chosen and the cell height measured at the narrowest part of the acinar wall, which is usually the part where the cell boundaries are best defined. The measure-

ments required from one animal were obtained from a single section of one of the thyroid "lobes."

STATISTICAL CONSIDERATIONS.

Analysis of variance.

The accuracy of the estimated mean cell height of the thyroids of a group of animals is affected by variation in the measurements. This variation is composed of two parts —variation between the acini in the same thyroid, the effect of which is reduced by measuring many acini in each gland, and variation between the thyroids of different animals, the effect of which can only be reduced by taking measurements from a large number of animals.

Let x = value of cell height from one acinus.
k = number of acini measured in each animal.
n = number of animals in the group.
p = mean value of x in one animal.
σa = standard deviation of the acinar measurements in one animal.

then $\sigma a = \frac{\Sigma (x-p)^2}{k-1}$.

Combining the data for the group $\sigma a = \frac{\Sigma (x_1 - p_1)^2 + \Sigma (x_2 - p_2)^2}{n(k-1)}$ . . . . .

Let θ = mean cell height which would be obtained by measuring all the acini in one animal.

then p = an estimate of θ from k acini.

This estimate has a standard error $= \frac{\sigma a}{\sqrt{k}}$.

$$p = \theta \pm \frac{\sigma a}{\sqrt{k}}$$

$$\text{then } \sigma p^2 = \sigma_\theta^2 + \frac{\sigma a^2}{k} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

This means that the variance of p is the sum of two variances, σ_θ^2 representing animal variance and $\frac{\sigma a^2}{k}$ representing acini sampling variance.

The estimated mean cell height of the group of n animals, M. $= \bar{p}$,

and the standard error of this estimate S.E.M. $= \frac{\sigma p}{\sqrt{n}}$;

$$\text{from (1) S.E.M.} = \frac{1}{\sqrt{n}} \sqrt{\sigma_\theta^2 + \frac{\sigma a^2}{k}} \quad . \quad . \quad . \quad (2)$$

$$= \sqrt{\frac{\sigma_\theta^2}{n} + \frac{\sigma a^2}{nk}} \quad . \quad . \quad . \quad . \quad (3)$$

From (3) it is evident that the total number of measurements, nk, is effective in reducing the contribution of acinar variation to the error of the estimated mean for the group. As k is made large the standard error S.E.M. approaches the limiting value $\frac{\sigma_\theta}{\sqrt{n}}$.

From (2) it is seen that this will be true when $\frac{\sigma a^2}{k}$ is small compared with σ_θ^2 for which the condition $k >> \frac{\sigma a^2}{\sigma_\theta^2}$.

For practical purposes there will be no advantage in making k greater than $10 \times \frac{\sigma a^2}{\sigma_\theta^2}$.

Measurement of variance.

Estimates of σa and σp have been calculated from the measurements of four groups of differently treated guinea-pigs and are given in Table I. From these values the estimates of σ_θ given in Table I have been calculated by the application of equation (1) above.

TABLE I.

Treatment of guinea-pigs.	n.	k.	$\sigma a(\mu)$.	$\sigma p(\mu)$.	$\sigma\theta(\mu)$.
1. Normal	15	50	$\pm$ 1·11	$\pm$ 0·84	$\pm$ 0·83
2. Injected thyrotropin	56	50	$\pm$ 1·20	$\pm$ 0·71	$\pm$ 0·69
3. ,, pot. iodide	16	50	$\pm$ 1·33	$\pm$ 0·50	$\pm$ 0·46
4. ,, thyroxine	10	50	$\pm$ 0·76	$\pm$ 0·56	$\pm$ 0·55

Application to measurement of thyroid response to thyrotropin.

The mean cell height of a group of animals is the mean value of p. This estimate has a standard error $\frac{\sigma p}{\sqrt{n}}$ found by treating p as a normal variate. It is not necessary to know either the number of acini measured in each thyroid or the standard deviation of the acinar measurements. The effect of increasing the number of acini measured is to diminish the standard deviation of p and therefore to decrease the standard error of the mean. As stated above there is no advantage in measuring more than 30 acini in each thyroid. The way in which the standard error is related to both n, the number of animals, and k, the number of acini measured in each thyroid, is illustrated by the table below, which shows the least value of k for different values of n which will give a standard error of the mean cell height less than $\pm$ 0·30μ in thyrotropin stimulated animals ($\sigma a = \pm$ 1·20μ, $\sigma_\theta = \pm$ 0·69μ).

Number of animals in group . . . n	5	6	7	8	9	10
,, acini measured in each thyroid . k	*	20	10	6	5	4

* Accuracy not attainable.

As a practical test of the prediction that useful results might be obtained by the measurement of only a small number of acini, we compared two groups of eight thyroid sections by measuring with the eyepiece micrometer four acini in each thyroid. One of the groups was normal, the other had received a weakly active pituitary preparation. The comparison was repeated by the projection method, measuring 50 acini in each gland.

By micrometer eyepiece, 4 acini per section : Cell height difference 2·56μ S.E. $\pm$ 0·70μ.

By projection, 50 acini per section : 2·97μ S.E. $\pm$ 0·42μ.

While useful results may be obtained from smaller numbers of measurements, we continue to measure 50 acini per section. This is accomplished rapidly enough by the projection method, and has the advantage that, without special efforts at random selection, the measurements are drawn from a sufficiently large area of the section to form a representative sample. By the more tedious eyepiece micrometer we would consider it an unwarranted expenditure of time to measure more than 20 acini per section.

The thyroid response to injected thyrotropin.

A preparation of thyrotropin from sheep pituitaries (flavianate prepared according to Evans, Meyer and Simpson, 1933) was used to obtain a response curve for three classes of guinea-pig: normal, iodide treated, and thyroxine treated. The sequence of injections has been given above in "Material and Methods: Animals." The thyrotropin was given in graded doses calculated on body-weight. Four animals were used for each dose level.

TABLE II.—*Mean Cell Height in Microns (4 Guinea-pigs in Each Group) after Thyrotropin Injection.*

Pretreatment of guinea-pigs.	Total dose, mg., of flavianate per 100 gm.						
	Nil.	0·5.	1·0.	2·0.	4·0.	8·0.	16·0.
Nil (normal)	6·9	—	8·7	9·4	11·4	12·2	13·3
Iodide	4·1	5·2	6·3	8·0	9·3	11·4	—
Thyroxine	3·4	4·6	5·9	7·7	9·5	10·9	—

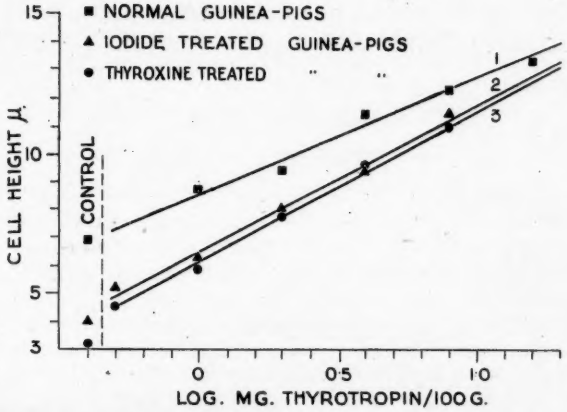

FIG. 2.—Relation between cell height and dosage of thyrotropin. Each plotted point represents the mean value of cell heights in a group of four animals receiving the same dose of thyrotropin. The lines are those for the regression equations as follows:
(1) For normal guinea-pigs: Log. mg. thyrotropin/100 g. = 0·236 × cell height — 2·01.
(2) For iodide treated guinea-pigs: Log. mg. thyrotropin/100 g. = 0·198 × cell height — 1·29.
(3) For thyroxine treated guinea-pigs: Log. mg. thyrotropin/100 g. = 0·186 × cell height — 1·13.

Cell height response.

The mean cell heights obtained are shown in Table II. In Fig. 2 these values are plotted against the logarithm of thyrotropin dosage. Over the range studied there is no significant departure from linearity. The three regression equations were fitted to the observations by the method of least squares. These equations are:

For normal guinea-pigs . Log. Mg./100 g. body-weight = 0·236 × C.H. — 2·01.
For iodide treated guinea-pigs . . . . . ,, ,, ,, = 0·198 × C.H. — 1·29.
For thyroxin treated guinea-pigs . . . . . ,, ,, ,, = 0·186 × C.H. — 1·13.

C.H. = Cell height in microns.

Thyroid weight response.

There was a positive correlation between thyroid weight and body-weight ($r = +0{\cdot}73$). The thyroid weights were therefore reduced to mg. per 100 g. body-weight, thus eliminating some of the variations. The thyroxine treated animals did not show any significant thyroid weight response. The results for the normal and iodide treated series are plotted in Figs. 3 and 4.

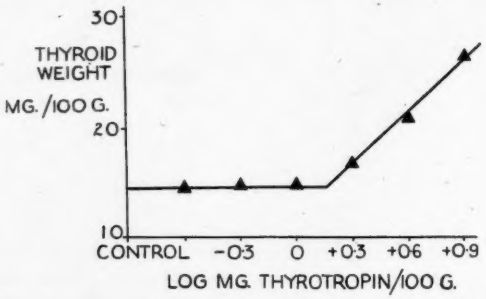

FIG. 3.—Relation between thyroid weight and dosage of thyrotropin in guinea-pigs pretreated with iodine. Small doses of thyrotropin did not produce any weight increase.

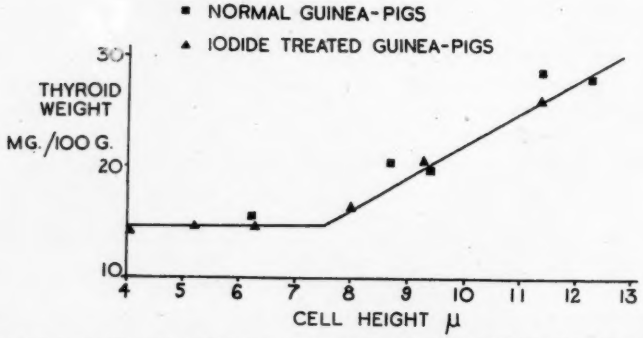

FIG. 4.—Relation between thyroid weight and cell height. The weight did not show an increase unless sufficient thyrotropin had been given to raise the cell height above about 8μ. The squares representing the not iodide pretreated animals indicate that these thyroids follow the same law, but since the initial cell height is greater, smaller doses of thyrotropin produced a weight increase.

DISCUSSION.

The most striking observation is that iodide and thyroxine did not diminish the thyroid cell response to thyrotropin. Indeed the response is greater in these treated animals and the slope of the response line consequently increased. The iodide treatment was so arranged as to leave but little iodide in the animals at the time the thyrotropin injections were made. The thyroxine, although continued during the thyrotropin injections, was given only in the small dosage of 10 μg. per 200 g. guinea-pig per day. Thus these results do not bear on the influence of large doses of iodide or thyroxine administered concurrently with thyrotropin for which an antagonist action has been described in the literature.

When the thyroid weight response is considered, it is found that the effect of iodide

has been to inhibit the response to the smaller doses of thyrotropin. This result can be reconciled with the cell height responses if we postulate that the weight does not begin to increase until the cell height has exceeded about 8μ. These effects of iodide have been confirmed by the results of assays of pituitary preparations on normal and on iodide treated animals.

Table III shows the responses produced by the injection of 2½ rat pituitaries per guinea-pig in normal and iodide treated guinea-pigs. There were eight guinea-pigs in each group.

TABLE III.

	Thyroid weight increase and S.E.	Cell height increase.
Normal guinea-pigs	16·20 ± 1·93 mg.	5·85 ± 0·39μ
Iodide treated	4·58 ± 1·64 mg.	6·84 ± 0·45μ

While the cell height increase has been augmented by the iodide treatment, the weight response is greatly diminished. It is considered that this effect is the result of the iodide treated thyroids being well filled with colloid when the thyrotropin injections are begun. The increase of the cells under stimulation is then offset by simultaneous loss of colloid. The greater cell height increase of the iodide treated thyroids is a consequence of their low cell height when the thyrotropin injections are begun, since at high thyrotropin dosages the cells in both types of gland tend to the same upper limit.

The use of the cell height response for the assay of thyrotropic activity.

Guinea-pigs with a low initial acinar cell height are the most useful for assays of thyrotropin by the cell height response. Under the conditions obtaining in New Zealand a preliminary iodide treatment is therefore advantageous, although this may not be necessary in environments naturally rich in iodine. We assume that the slope and position of the response curve is determined solely by the initial cell height. In doing preliminary assays we use eight guinea-pigs for each preparation tested. The substances tested are injected intraperitoneally in five equal doses on consecutive days. All animals in the group are given the same absolute dose regardless of their body-weight. A group of eight animals not receiving thyrotropin injections serves for a control of the initial cell height. If this agrees with the value found when the response curve was constructed (i.e. 4·1μ for iodide treated animals), the regression equation is used to calculate the activity of the test dose in terms of an arbitrary unit equivalent to 1 mg. of our flavianate preparation. Thus, for iodide-treated guinea-pigs—

Log. U = 0·198 C.H. + mean Log. W. − 3·29.
where U = activity of test dose in units equivalent to mg. of flavianate.
C.H. = mean cell height of group in micra.
Mean Log. W = mean of the logarithms of the weights of the guinea-pigs in grammes.

The standard error of the mean cell height of a group of eight of our guinea-pigs is approximately ± 0·30μ. The standard error of log. U is therefore approximately ± 0·06, corresponding roughly to an error in U of ± 15 per cent. In addition there are errors involved in the use of constants derived from the response curve of a different batch of guinea-pigs. Where precise results are required the potency of the test

substance must be checked against doses of a standard preparation. In our experience, however, the response curve has proved sufficiently stable to enable the use of results obtained from the preliminary assays in investigations of pituitary physiology.

SUMMARY.

1. Thyroid acinar cell heights can be conveniently measured by projection of sections on to a white screen at a magnification of × 1000 and the use of a transparent scale.

2. From a study of acinar and thyroid variation it appears to be unnecessary to measure more than about 20 acini from each animal.

3. Treatment with iodide or thyroxine increases the cell height response to injected thyrotropin. At high thyrotropin doses the cell heights for iodide, thyroxine, and "normal" animals converge to the same point.

4. Treatment with iodide or thyroxine greatly diminishes the thyroid weight response to injected thyrotropin. This is due to colloid loss offsetting the effect of cell hypertrophy.

5. The iodide treated guinea-pig is a suitable animal for assays of thyrotropic activity by cell height response.

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BASAL METABOLISM OF THE ALBINO RAT FED ON GOITROGENIC DIET.

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POSITIVE goitrogenic agents have been demonstrated in cabbage (Chesney, Clawson and Webster, 1928), in soya bean (Sharpless and Hopson, 1940), and in seeds of the Brassicae family (Hercus and Purves, 1936). The metabolic rate of rabbits fed on goitrogenic diet was investigated by Chesney, Clawson and Webster and found to be subnormal. A similar result was found for the rat on soya bean diet by Sharpless and Hopson (1940). J. L. Malcolm (1937) in this laboratory investigated the metabolism of rats on Brassicae seed diets and found it normal or slightly depressed. MacKenzie and MacKenzie (1943) and Astwood, Sullivan, Bissell, and Tyslowitz (1943) have reported the goitrogenic action of sulphonamides in the rat, and have found that the thyroid hyperplasia which these substances produce is accompanied by lowered metabolic rates.

This paper reports further measurements of metabolism of rats on Brassicae seed diets. Since the failure to demonstrate an increased metabolism was at variance with the marked enlargement and histological activity of the thyroid glands, further experiments were made to determine if the Brassicae seed factor modifies the action of thyroxine administered by injection.

METHODS.

Measurement of Oxygen Consumption.

The apparatus was a closed circuit type with accurate temperature control. Before use the apparatus was checked by the physiological test of Benedict (1930). The oxygen consumption in each animal was measured for a period of fifty minutes on three separate occasions.

All the conditions for measurement of rat metabolism as laid down by Benedict and Macleod (1928) and Horst, Mendel and Benedict (1934) were closely followed. Albino rats of the Wistar strain were used. The composition of the Brassicae seed diet was as previously described by Kennedy and Purves (1941).

RESULTS.

Oxygen Consumption of Rats fed on Goitrogenic Diets.

In early experiments steamed white mustard seed was found to possess goitrogenic properties. Six rats were fed on a diet containing a high proportion of this seed for three weeks. In the results shown below oxygen consumption is expressed per unit of rat weight per minute, two hundred grammes being chosen as a convenient standard. The oxygen consumption of numbers of normal rats of the Wistar strain was measured, the figures comparing closely with those already published by Benedict and his co-workers. After the above six rats had been measured, their thyroids at autopsy were found to be histologically activated and to be hypertrophied to several times the usual weight.

In another similar experiment a group of five rats were fed a diet containing a proportion of Chou moellier seed during a period of four weeks. The metabolism in this group was also measured.

In a further experiment an attempt was made to prevent the thyroid changes by daily injection of 1 mg. of iodine as potassium iodide. From a group of ten rats so treated for 30 days and simultaneously fed on rape seed diet, three were chosen as representative of the group and oxygen consumption measured. The results of these experiments are shown in Table I. It was also found that animals fed on rape seed diet alone failed to show significant variation from the accepted normal figures in oxygen consumption.

Oxygen Consumption of Thyroidectomized Rats.

Griesbach, Kennedy and Purves (1941) have already shown the rapid histological hyperplasia and hypertrophy produced by the action of the diets. They have also demonstrated that the mediation of the thyrotropic hormone of the pituitary is necessary for the thyroid hyperplasia to be developed. As the metabolism of these rats fed on the diets falls within normal limits we assume that the hyperplastic thyroid is producing thyroid hormone sufficient for bodily needs. The histological picture, however, may lead the observer to expect an elevated metabolism. It is necessary, therefore, to exclude the possibility that should the thyroid hormone be produced in excess by such a gland, its peripheral action may be inhibited by a factor in the diet. It was planned to discover whether in a thyroidectomized animal the action of thyroxine is inhibited when such an animal is fed on the activating diet.

TABLE I.—*Oxygen Consumption of Rats Fed on Goitrogenic Diets.*

No. of rats.	Diet.	Weight of rats (g.).	Oxygen consumption (c.c./200 g./min.). Mean.	Oxygen consumption (c.c./200 g./min.). Range.	Oxygen consumption. Control group. Mean.	Oxygen consumption. Control group. Range.
6	Steamed white mustard seed	70–90	6·16 c.c.	7·0–5·6 c.c.	6·11 c.c.	7·2–5·7 c.c.
5	Chou moellier seed	70–89	5·63 ,,	5·84–5·6 ,,	5·83 ,,	5·7–6·1 ,,
3	Rape seed; 1 mg. iodine daily	60–64	6·11 ,,	6·04–6·28 ,,	5·99 ,,	5·76–6·46 ,,

Male rats were thyroidectomized and those animals selected which showed a constant subnormal metabolic rate. One group of five animals was placed on rape seed diet and a control group of three animals retained on normal diet. After ten days the animals received a single injection of thyroxine equivalent to 1 mg. per 100 gm. body-weight. The maximum increase of metabolic rate for the rape-seed diet rats averaged 41 per cent., for the control diet 38 per cent. It was concluded that the diet did not influence the response to thyroxine administered in a single large dose.

Meyer and Wertz (1939) showed that when the thyroidectomized rat has its metabolism raised above the normal level, its sensitivity to increased thyroxine administration is lessened as compared with its previous response below that level. Moreover, thyroxine administered in small divided doses is more effective in raising the metabolism than when given in a single dose large. A second thyroidectomy experiment was designed with the object of maintaining the metabolic rate of the thyroidectomized animal at about the normal level by small daily injections of thyroxine. A group of male rats, having been thyroidectomized at the age of five weeks, were allowed eight weeks to attain a stable metabolic level. Four were then placed on rape seed diet and six on control diet. Eight days later daily subcutaneous injection of thyroxine commenced, both groups of rats being given a dose of 7·5 μg. per 100 g. of body weight. During the following 22 days the injections continued and metabolic reactions were studied.

After a pause of 14 days, in which the animals reached a stable metabolic level again, all the animals were given a further 14 days' course of thyroxine injections of a dose equivalent to 25 μg. per 100 g. of rat weight. The metabolic rate rose a little higher for both groups and was also studied during the nine days occupied for the descent. At autopsy all the animals were found to be fully thyroidectomized.

The results are depicted in Fig. 1.

No significant difference is seen in the oxygen consumption of the groups of rats fed on the two diets. Also when the injections of thyroxine had ceased the rate of descent of oxygen consumption back to normal was the same for both groups of animals.

DISCUSSION.

The work of Kennedy and Purves (1941) has demonstrated the powerful goitrogenic agent which exists in the Brassicae seed diets, and has shown that neither the presence of adequate iodine in the diet nor the daily injection of excess iodine is capable of preventing the production by the diet of thyroid hyperplasia and hypertrophy. It is seen that the metabolism of normal rats fed on the Brassicae seed diets lies within normal limits, so that the hypertrophied thyroid is still secreting

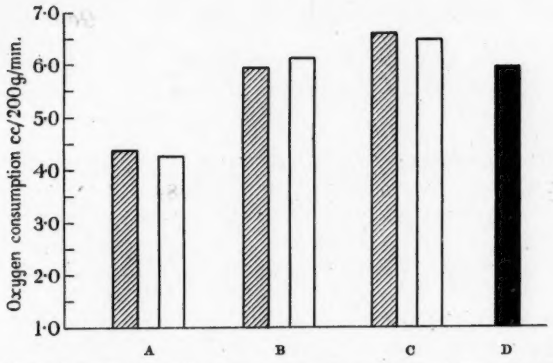

FIG. 1.—Effect of daily thyroxine injections on oxygen consumption of thyroidectomized rats fed on seed and control diets.

A. Thyroidectomy. Level of metabolism.

B. Thyroxine. 7·5 mg./100 g. per day.

C. Thyroxine. 25 mg./100 g. per day.

D. Group of normal rats of same weight.

▨ Group on seed diet, thyroidectomized.

☐ Group on control diet, thyroidectomized.

enough hormone for bodily needs. From the data obtained from thyroidectomized animals fed on the Brassicae diet there does not appear to be any factor in the diet which inhibits the action of thyroxine on the peripheral cell. It is also known that the presence of the thyrotropic hormone is necessary for the changes to take place in the thyroid when the animal is fed the diet. If it is assumed from the data of the above workers that the thyrotropic hormone is being produced in excess due to stimulation of the pituitary by the diet, we are led to the conclusion that the diet must also at the same time counteract the complete chemical maturity of the hypertrophied thyroid cell.

SUMMARY.

The oxygen consumption of albino rats fed on the "goitrogenic" principle of Brassicae seeds is found to lie within the limits accepted as normal for animals of the same weight and sex.

The diet itself was found to contain no factor inhibiting the action of injected thyroxine on the peripheral tissues of the thyroidectomized rat.

It is concluded that the hyperplasia produced by these seeds is not accompanied by increased thyroxine secretion.

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THE ENZYMES OF HEALING WOUNDS. I. THE DISTRIBUTION OF ALKALINE PHOSPHOMONOESTERASE IN EXPERIMENTAL WOUNDS AND BURNS IN THE RAT.

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DURING the process of wound healing much synthetic activity must take place in the region of repair. It therefore seems probable that a study of the enzymes of healing wounds will provide information, important from both the practical point of view of wound healing, and from the point of view of discovering the biological significance of the various enzymes concerned. The present study deals with the alkaline phosphatase which splits off phosphate from such substances as hexose phosphate, glycerophosphate, and phenol phosphate (Foley and Kay, 1936), with optimal activity at alkaline pH. The distribution of this enzyme in small experimental wounds and burns at different stages of healing has been studied by the histochemical methods of Takamatsu (1939) and Gömöri (1939); where possible the histochemical results have been checked by the usual chemical methods for determining phosphatase activity.

The principle of Gömöri's technique is as follows: Phosphate freed by phosphatase is precipitated immediately as calcium phosphate. The precipitate of calcium phosphate is converted to cobalt phosphate, and this on treatment with a soluble sulphide forms cobalt sulphide, giving a brownish-black stain which is believed to be localized in the immediate vicinity of the enzyme.

MATERIAL AND METHODS.

Material.

Rats aged 9–18 months were used, one wound or burn being made on each animal. The histological observations were made on 16 standard wounds and 10 burns, and the chemical estimations of phosphatase on 6 standard wounds.

Surgical Methods.

The standard wounds were made under ether anaesthesia by the method described by Dann, Glücksmann and Tansley (1941). An area of dorsal skin was closely shaved and wiped with 80 per cent. alcohol. A circle was cut in the shaved area with a rotating cylindrical blade 6 mm. in diameter, and the skin, including the panniculus carnosus, was excised with fine scissors. A patch of sterile oiled silk was laid over the wound and fixed to the skin with two strips of adhesive plaster, a strip of cotton-wool was wrapped round the body so as to cover the oiled silk and the whole was held in position by a plaster bandage round the thorax (Glücksmann and Dann, unpublished). The plaster prevents the rats from interfering with the wound, and if well padded with

cotton-wool it seems to cause the animals no discomfort, and they make no serious attempt to remove it.

Burns were made under ether anaesthesia with a soldering iron 1·5 mm. in diameter applied for about one second. This produced a small, shallow burn. In other respects the technique was exactly the same as for the wounds.

Histological Methods.

The rats were killed at various intervals from 24 hours after operation, and an area of skin containing the wound or burn was removed and fixed in 80 per cent. alcohol for 5–7 hours. It was then transferred to absolute alcohol for 24 hours, cleared in cedar wood oil and embedded in paraffin wax for about 9 hours. The blocks were cut within a day of being made. The thickness of the sections was 10μ.

The rest of the technique was exactly as described by Gömöri, except that the slides were incubated in the salt solution containing sodium glycerophosphate for 24 hours instead of for 2–3 hours as this author recommended. Preliminary experiments had shown that this longer incubation in the glycerophosphate solution emphasized the contrast between positively and negatively reacting regions, and demonstrated a moderate positive reaction in areas which appeared almost negative with the shorter incubation. On the other hand, slides incubated for 2–3 hours have a special value for comparison with those incubated for 24 hours, as they indicate very clearly where the highest concentrations of phosphatase are situated.

One slide from each block was stained with Ehrlich's haematoxylin and erythrosin, four slides were incubated in the solution containing sodium glycerophosphate, and two controls were incubated in salt solution from which the sodium glycerophosphate had been omitted. All six slides were incubated simultaneously for the same length of time. During subsequent treatment with cobalt nitrate and ammonium sulphide the sides were run through the solutions in pairs, one of each pair being an experimental and the other a control slide, so that the results should be strictly comparable.

Phosphatase Estimation.

When the six standard wounds were made for this purpose, the circle of skin drilled out of each rat was fixed in 80 per cent. alcohol and used for a control estimation. Six days after operation the rats were killed. When a scab was still attached to the scar it was removed, all the scabs were collected in one tube of 80 per cent. alcohol and their joint phosphatase activity estimated. After removal of the scab, the wound area was excised with the drill, but owing to the contraction of the wound only part of this circle of skin consisted of regenerated tissue. As a second control, an area of undamaged skin near the wound was also removed with the drill. The individual pieces of tissue were fixed in 80 per cent. alcohol for about five hours, roughly dried on filter-paper, weighed, and ground to a pulp with a little acid-washed sand. The ground tissue was washed into a tube with 5 ml. of a mixture of equal parts of 2 per cent. sodium glycerophosphate, 2 per cent. calcium nitrate and 2 per cent. sodium veronal.* This mixture was incubated at 37° C. for 20 hours. After incubation the protein was precipitated, and any insoluble phosphate brought into solution by addition of 20 per cent. trichloracetic acid. After filtration the inorganic phosphate was determined by the method of Fiske and Subbarov (1925), and expressed as milligrams of PO_4 split off per gramme of tissue per second. For control experiments the piece of tissue was heated at 100° C. for five minutes before grinding.

* Inorganic phosphate is an inhibitor of phosphatase, but with this incubation mixture is precipitated as soon as formed, so that there is no progressive inhibition due to liberated phosphate.

RESULTS.

Normal Skin.

As reported by Gömöri, sections of normal skin treated for the demonstration of phosphatase react very little. In the rat the extreme proximal tips of the hair follicles react intensely, the epithelial cells being almost black, and the sebaceous glands are also strongly positive. A few of the superficial capillaries and some of the perivascular fibroblasts are fairly dark, but the rest of the dermis and the epidermis are almost colourless. The panniculus carnosus shows the spurious staining mentioned by Gömöri, which also appears in the controls.

Wounds.

Twenty-four hours after operation the wound is largely filled with fibrin and is usually covered at least partly by a scab. The fibrin and the tissue surrounding the wound are heavily infiltrated with leucocytes. At this stage only the polymorphs gave an intense reaction for phosphatase, their nuclei being usually black and their cytoplasm very dark grey. Consequently the scab (fig. 1), owing to its high content of dead and dying polymorphs, is much the most conspicuous object in preparations made by Gömöri's method. The nuclei of the migrating monocytes and lymphocytes are also positive, the nucleoli, chromatin granules and nuclear membrane being nearly black as if stained with iron haematoxylin, but the nuclear reaction is far less intense than in the polymorphs. Gömöri (1943) noted that leucocytes in tubercular lesions of the lungs are strongly positive.

At the extreme margin of the day-old wound a few capillaries have just begun to invade the fibrin clot, and by the end of the second day (figs. 1 and 4) the vessels have pushed well into the cavity, though the interior of the fibrin is still avascular (for details of the vascularization process see Hughes and Dann, 1941). The endothelium of many of these invading capillaries is strongly positive. The epidermis round the margin of the wound has become thickened, and the nuclei are much darker than those of the normal epithelium.

Much of the fibrin in the wound has been resorbed by the third day, vascularization is well advanced and many fibroblasts have wandered into the cavity. The nuclear

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DESCRIPTION OF PLATES.*

Abbreviations.—ep., regenerating epidermis; *p.*, polymorphs; *r.c.t.*, regenerating connective tissue; *sc.*, scab.

All the preparations were made by Gömöri's method for the demonstration of phosphatase. The phosphatase is believed to be localized in the regions stained black.

FIG. 1.—Section of a wound fixed 2 days after operation. The wound is filled with fibrin and infiltrating leucocytes, of which the polymorphs give the strongest reaction. Note the intense reaction of the scab resulting from its very high polymorph content. (× 14.)

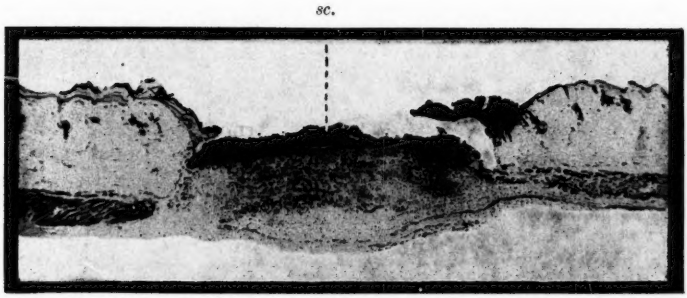
FIG. 2.—Section of a wound fixed 5 days after operation. The cavity is now filled with regenerating connective tissue giving a very strong phosphatase reaction. (× 14.)

FIG. 3.—Section of a small burn fixed 7 days after operation. The damaged tissue, heavily infiltrated with the remains of polymorphs, has been sloughed though it remains adherent. Epithelialization beneath the scab is complete and a shallow layer of intensely reacting young connective tissue has formed beneath the new epithelium. (× 21.)

FIG. 4.—Part of the same section of a 2-days-old wound as that shown in fig. 1. Note the intensely reacting polymorphs in the fibrin clot; the black region at the top of the photograph is the deeper portion of the scab. (× 290.)

FIG. 5.—Part of the same section of a 5-days-old wound as that shown in fig. 2. Beneath the regenerating epithelium are seen the intensely reacting cells and fibres of the regenerating connective tissue. (× 290.)

* The photomicrographs were made by Mr. V. C. Norfield, Senior Assistant at the Strangeways Research Laboratory.



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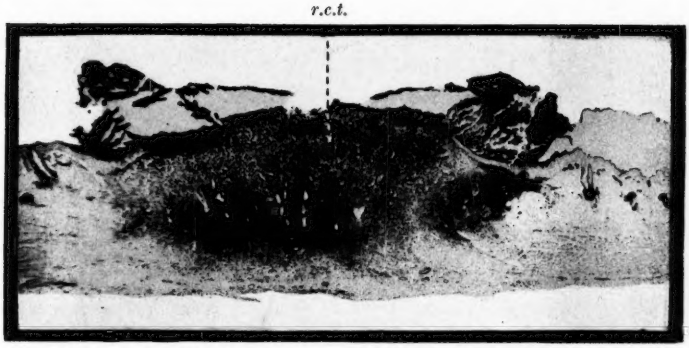

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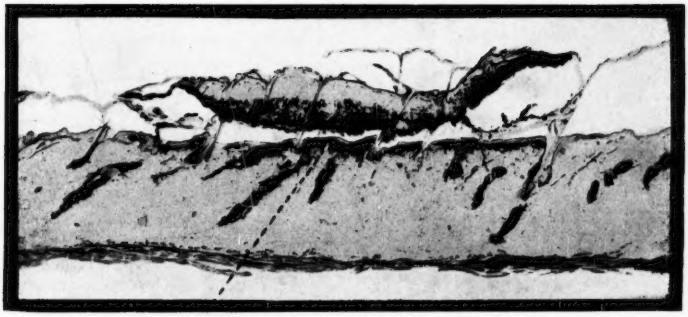

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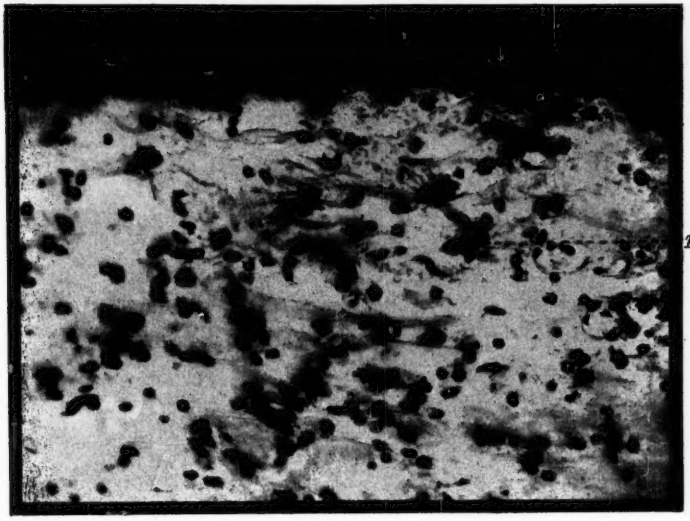

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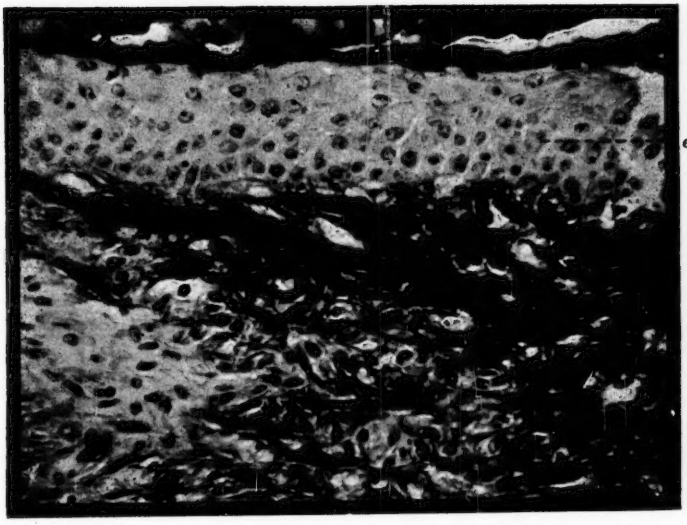

FIG. 5.

reaction of these invading fibroblasts varies somewhat, but is much stronger than in the fibrocytes of the uninjured dermis ; the cytoplasm shows little darkening.

About the fifth day (figs. 2 and 5) there is a spectacular increase in the reactivity of the regenerating tissue. If sections treated by Gömöri's method are viewed with the naked eye, the wound area is seen as a dark patch contrasting sharply with the almost colourless tissue on either side. Microscopic examination shows that this reaction is most intense in the regenerating collagen which Dann, Glücksmann and Tansley have described as uniting " the cut edges of the fibre bundles of the original collagen layer " and " forming a deep collar encircling the wound." This collar consists of a compact mass of spindle-shaped fibroblasts and fine intercellular fibres orientated parallel with the surface of the skin, and it is pierced by many blood vessels running roughly at right angles to the cells and fibres (Hughes and Dann, 1941). The nucleus and cytoplasm of the fibroblasts, the intercellular fibres and most of the blood vessels are very dark, in places almost black (fig. 5). The chromosomes of the dividing cells in the collar also react very strongly (*cf.* Willmer, 1942), but this does not seem to be characteristic of all mitotic figures ; those in areas like the regenerating epidermis, where the general reaction of the tissue is much less intense than in the developing collagen, have much paler chromosomes. The collagenous collar does not occupy the whole thickness of the wound at this stage, and the spaces above, below and encircled by it are filled by a richly vascular tissue in which the cells and vessels have no regular arrangement. In this latter tissue the fibrocytes and capillaries react almost as intensely as those of the collagenous collar, but the intercellular material, which consists largely of partly resorbed fibrin, reacts much less than the young collagen fibres of the collar. At the cut edge of the panniculus carnosus the musele is beginning to regenerate, and the newly formed myoblasts show a slight increase in phosphatase reaction as compared with the old tissue which, as stated above, is almost negative.

The wound is fully epithelialized by the 7th day, and the irregular tissue above, below and within the collagenous collar has been almost or entirely replaced by orderly, intensely reacting layers of fibroblasts and young collagen fibres like those of the original collar.

The intense phosphatase reaction begins to decline about the 11th day. This decline begins in the deeper part of the wound and gradually spreads towards the surface, so that vertical sections show a colour gradient from a strongly positive region beneath the epidermis to a comparatively colourless zone at the level of the panniculus.

In a scar fixed on the 19th day (the oldest examined) the phosphatase content of the regenerating tissue, as indicated by Gömöri's method, appears almost normal except near the margin of the wound area, where the tissue immediately beneath the epidermis still reacts quite strongly. Some of the capillaries in the interior of the wound are also intensely positive. In the particular specimen examined a tiny secondary injury had somehow been produced in the new epithelium, and immediately beneath this minute lesion the intensely dark colour characteristic of young regenerating connective tissue has reappeared.

Burns.

In a one-day burn, as in a wound at this stage, the only strongly positive reaction in the damaged area is due to the invading leucocytes, particularly the polymorphs. A tract of epidermis has been killed by the iron and, like the scab of a wound, appears almost black owing to dense infiltration with polymorphs. As stated above, the burns used for these observations were shallow, and only the superficial part of the dermis is dead ; this, like the overlying epithelium, contains many polymorphs. The deeper

parts of the hair follicles and of the dermis survive. The capillaries in the living tissue surrounding the burn are dilated and some are strongly positive; numerous leucocytes are wandering through the endothelium.

After two days the dead connective tissue and epidermis are almost completely split off from the underlying healthy tissue by the regenerating epithelium, which spreads over the surface of the undamaged dermis both from the margin of the burn and from the surviving part of the hair follicles. The nuclei of the new epithelium and also of the surviving portion of the hair follicles react more strongly than those in the uninjured skin surrounding the burn.

The burn is completely epithelialized by the 3rd day, though the scab of dead tissue remains attached for some time (*cf.* fig. 3). The dermis also begins to regenerate beneath the new epithelium, as indicated by the gradual accumulation in this region of fibroblasts which give a slightly positive reaction with Gömöri's method.

It is not until about the 6th day, however, that any marked change is seen. A thin, but intensely reacting layer of young connective tissue has then formed beneath the epidermis, and in section is seen as a thick black line under the epithelium. This line becomes broader as regeneration proceeds (fig. 3), and sections stained with haematoxylin and erythrosin show that it consists of very cellular young fibrous tissue exactly like that seen in healing wounds from about the 5th day.

The reactivity of the regenerated connective tissue has begun to decline by about the 12th day, and in a burn fixed after 17 days is only just detectable.

Controls.

Histological controls.

The distribution of the positively reacting tissue in the normal skin and in wounds and burns was carefully checked by comparing the experimental sections with the control series incubated in saline without sodium glycerophosphate. In the controls, the keratin, some of the hairs and, less markedly, the panniculus carnosus stain fairly darkly, but the rest of the section is an undifferentiated, pale greenish yellow throughout.

Control estimations.

Although the histochemical evidence for an increase in phosphatase content during healing seemed very clear, it was thought advisable to obtain an independent check on this conclusion. For this, pieces of skin containing five-day wounds (at which time the phosphatase content has reached a maximum, to judge from the histological preparations), were treated as described under "Methods," the phosphatase content of the wounded area being estimated in terms of milligrams of chemically determined phosphate split off in a standard time. The results shown in Table I fully confirm

TABLE I.

a. Animal.	b. Normal skin.	c. Healing area.	c/b.
1	$10{\cdot}2\times10^{-6}$	$29{\cdot}5\times10^{-6}$	2·9
2	$6{\cdot}9\times10^{-6}$	$16{\cdot}0\times10^{-6}$	2·3
3	$9{\cdot}4\times10^{-6}$	$27{\cdot}0\times10^{-6}$	3·0
4	$8{\cdot}2\times10^{-6}$	$13{\cdot}0\times10^{-6}$	1·6
5	$9{\cdot}5\times10^{-6}$	$29{\cdot}5\times10^{-6}$	3·1
Pooled scabs	..	$>318{\cdot}0\times10^{-6}$	(>36·0)

The table shows the phosphatase activity of normal and healing skin in terms of milligrams of PO_4 liberated per second per gramme of tissue.

the histological findings. The phosphatase content of the skin fragment containing a healing area is in all cases substantially greater than in a corresponding weight of normal skin, although, as stated above, only part of the fragment consists of regenerating tissue. The high figure for the pooled scabs—more than 36 times the average phosphatase content of normal skin—also accords well with the histological observation that the scab is much the most strongly reacting part of the wound.

The effect of activating and inactivating substances on the phosphatase reaction.

A further possible source of error in the comparison of normal and healing skin resides in the possibility that normal skin may contain much inactive enzyme, which is activated by substances, such as SH compounds, liberated by the proliferating tissue. The results presented above do not discriminate between a difference in *total* alkaline phosphatase of normal and healing skin, and a difference in the *active fraction* of the phosphatase in the two cases. To check this source of error, skin sections were incubated with a number of substances likely to activate and inactivate phosphatase. The histological results, which are shown in Table II, were checked by chemical estimation of inorganic phosphate set free by ground skin ; the results of these experiments are shown in Table III. Although some substances activate the enzyme, this in no case caused phosphatase to become evident in sites which were devoid of phosphatase before treatment with the activator. The effect of activators was merely to increase the activity in sites which still had activity in the absence of activators.

TABLE II.—*Activation and Inactivation of Phosphatase in Skin Sections by Various Substances.*

Substance.	Activation.	Inactivation.
2% $MgCl_2$	(+)	..
0·4% oxidized glutathione	+	..
0·2% iodoacetate	+	..
0·4% reduced glutathione	..	+
0·4% cyanide	..	++
0·2% vitamin C	..	+
0·4% thioglycollate	..	++
ammonium sulphide	..	+
M/4 glycine	..	+

Comparison of Tables II and III shows agreement between the histochemical and the chemical estimations of phosphatase activity, except in the case of glycine. Histochemically glycine, as found by Kabat and Firth (1941), appears to inhibit phosphatase,

TABLE III.

Substance (concentration as in Table II).	Per cent. of original activity.
None	100
$MgCl_2$	210
Oxidized glutathione	160
Iodoacetate	120
Reduced glutathione	80
Cyanide	15
Thioglycollate	<10
Ammonium sulphide	20
Glycine	110

i.e., the amount of calcium phosphate precipitated in a tissue section is reduced. But direct chemical estimation of the phosphate set free shows that glycine causes no significant change in the amount liberated. The misleading result obtained by the histochemical method is probably due to the fact that the solubility of calcium salts is increased by aminoacids (Lehmann and Pollak, 1942), so that phosphate released in a section is not precipitated, and may diffuse out of the section, when glycine is present.

DISCUSSION.

Our results have shown that during the healing of a skin wound or burn, the regenerating tissue has a much greater alkaline phosphatase content than normal skin. The phosphatase reaction is particularly intense during the formation of the intercellular fibres. So far, the intracellular functions of this enzyme have not been discovered, and we have no knowledge of the particular aspects of synthesis with which it is concerned. However, it seems unlikely that the correlation between collagen formation and phosphatase appearance is fortuitous, and we incline to the view that the phosphatase is connected, directly or indirectly, with the metabolic processes more intimately concerned in the laying down of collagen. Bourne (1943) has noted a similar correlation between phosphatase activity and fibre formation in regenerating bone. Whilst it seems probable that the phosphatase associated with freshly formed collagen is derived from the cells of the regenerating region, there remains the possibility that the enzyme is selectively adsorbed upon the newly formed fibres from the interstitial fluid circulating through the regenerating area, and may therefore be derived in part at least from some other source or sources.

Under intracellular conditions the oxidation-reduction potential and pH in the vicinity of the enzyme must both be involved in determining the level of its activity. Normally, to judge from our observations on the effect of reduced glutathione, the intracellular phosphatase activity is likely to be far from optimal, since the enzyme must be largely in the reduced (inactive) form, and is some distance from its optimum pH. This view is subject to correction, however, since we have at present little knowledge of the pH and oxidation-reduction potential of localized regions of the cell.

The cytological distribution of the calcium phosphate precipitated as a result of phosphatase activity is also of some interest. We are not satisfied, however, that the localization of this precipitate on the cell nuclei and on the newly formed collagen fibres necessarily indicates that the tissue phosphatase is also localized in precisely the same manner. We hope in a later communication to discuss this phenomenon in more detail.

SUMMARY.

1. Changes in skin alkaline phosphomonoesterase have been studied during the healing of experimental wounds and burns in rats.

2. During the early stages of repair only the invading polymorph leucocytes give an intense staining reaction for phosphatase. The scab, owing to its high polymorph content, is the most deeply stained part of the wound area.

3. The regenerating connective tissue reacts more strongly than the normal dermis. The intensity of its reaction increases as healing advances and reaches a maximum when the formation of collagen fibres begins. At this stage the fibroblasts, intercellular fibres and capillaries stain deeply.

4. Skin phosphatase is activated by oxidized glutathione, magnesium and iodoacetate. It is inactivated by reduced glutathione, thioglycollate, ammonium sulphide and ascorbic acid. It is not inactivated by glycine, but an apparently negative histochemical reaction may be obtained when glycine is present.

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NUTRITIONAL STUDIES OF BACTERIAL VARIATION. I. RESISTANCE TO PANTOYLTAURINE IN NATURALLY-OCCURRING AND EXPERIMENTALLY PREPARED STRAINS.

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THE phenomena of drug resistance limit the value or manner of administration of many chemotherapeutic agents. Resistant micro-organisms commonly maintain their fastness for long periods, while retaining the normal invasiveness of the parent organism. Sulphonamide-resistance has received special attention following the widespread use of these compounds, and minor epidemics of resistant organisms have been reported; resistance induced by one sulphonamide has been found to extend completely or partially to all sulphonamides. The antibacterial agent, pantoyltaurine, is a sulphonic acid derivative, whose structure was partly based on that of sulphanilamide, and was suggested by one of the few rules at present available for designing chemotherapeutic agents (Fildes, 1940; McIlwain, 1942*a*). The resistance of bacteria to pantoyltaurine has now been investigated. The first aim of this study has been to find whether organisms resistant to pantoyltaurine exhibited cross-resistance to sulphanilamide, or, more generally, whether the method of designing drugs which is represented by pantoyltaurine necessarily leads to cross-resistance. This involved the isolation of strains of *Streptococcus haemolyticus* and *Corynebacterium diphtheriae* which were resistant to pantoyltaurine. The mode of action of pantoyltaurine is understood to a greater extent than are those of most chemotherapeutic agents, and as a second aim the relevant biochemical behaviour of the normal

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and resistant strains has been studied in an attempt to find the nature of the drug-resistance. The inhibitory action of pantoyltaurine, both *in vitro* and *in vivo*, is antagonized by pantothenate, a growth-essential which is related to it in structure (McIlwain and Hawking, 1943); special attention has accordingly been given to the relations of the different strains to pantothenate and to its constituents, β-alanine and pantoate (β.β-dimethyl-α.γ-dihydroxybutyrate). Organisms respond to mixtures of different concentrations of pantothenate and pantoyltaurine in such a way that the effect of a given ratio of concentrations of the two compounds is roughly constant. The behaviour of an organism in this connection has therefore been expressed by the effects of different values of that ratio, or by the effects of various concentrations of pantoyltaurine in the presence of fixed concentrations of pantothenate.

EXPERIMENTAL.

Technique.

The organisms were maintained on serum-agar or blood-agar slopes and in testing were grown in liquid media, whose pantothenate content could be controlled by direct addition of the compound. In investigating the reactions of strains to pantothenate and pantoyltaurine, the general arrangements and materials were those previously described (McIlwain; 1942*b*, *c*) employing 2-fold dilutions of the reagents whose effects were being examined, and inocula of about 1000 cells in test-solutions of 10 ml. The basal media throughout contained acid-hydrolysed casein, which contributed about $5 \times 10^{-8}M$ β-alanine to the final test-solutions. In addition, the group of growth-factors added to media for streptococcal growth (but not for that of *C. diphtheriae*) contributed a further $2 \times 10^{-5}M$ β-alanine.

Before attempting the preparation of resistant strains, the parent organisms, which were already pure by ordinary criteria, were plated serially from 4 to 6 times from known dilutions of suspensions from single colonies. The number of cells in these inocula were approximately known, and never greatly exceeded the number of colonies growing; the resulting organisms were characterized by their haemolysis and antigenic group in the case of streptococci, or by starch fermentation and appearance in the case of *C. diphtheriae*. Resistant strains were prepared by repeatedly subculturing the organisms in the presence of pantoyltaurine. Each subculture was made to a series of tubes containing a range of concentrations of pantoyltaurine and the minimal concentration of pantothenate which gave full growth; this was usually $10^{-7}M$. The tubes of highest pantoyltaurine content, in which growth had occurred in the course of 5 days, were used as inocula for further series. From 12 to 20 such subcultures were made, and when growth was found to occur in the presence of progressively higher concentrations of pantoyltaurine, these were increased while maintaining the same pantothenate level. The successive inocula were large, and preferably taken from tubes in which growth had been delayed by the drug. The organisms used as inocula were at each subculture, or at alternate subcultures, grown on solid media and examined microscopically. It was necessary to confirm their purity in this way, as the inhibitor usually caused morphological distortion of the organisms. When the final strains were compared with the original organisms in Group or Type, no changes were found.

The term " training " is used here to indicate any process of attempting to change an organism by growing it under unusual conditions. The conditions of development of trained strains are indicated by suffixes to the names of their parent strains, as in $G1_{PT}$ (p. 208).

Sulphonamide-resistant organisms.

Eleven strains were investigated ; they included haemolytic streptococci isolated from wound infections (Francis, 1942), and are compared in Table I, A, with normal streptococci. The susceptibilities of the sulphonamide-resistant organisms to pantoyltaurine were little different from those of the normal strains. The sulphanilamide susceptibilities of all organisms were also tested under the same conditions of growth, and the marked differences which are recorded were found. The ratio of pantoyltaurine to pantothenate concentrations which were necessary for bacteriostasis were usually determined with only one or two concentrations of pantothenate, but in the cases of two sulphonamide-resistant organisms a series of concentrations were examined, and the competitive nature of the inhibition, previously reported with normal streptococci (McIlwain, 1942*b*), was found also in the sulphonamide-resistant organisms. All the sulphonamide-resistant streptococci required pantothenate for growth, and in the 8 cases so examined, the minimal concentration for full growth was about $5 \times 10^{-8}M$; no visible growth occurred with $10^{-9}M$.

Pantoyltaurine resistance.

Natural.—In contrast to the findings with Group A organisms, a wider range of natural streptococcal types showed great differences in their susceptibilities to pantoyltaurine and in their needs for pantothenate (Table I, B). The concentrations of pantoyltaurine necessary for inhibition of different *viridans* strains in the presence of a constant concentration of pantothenate varied from that typical of Group A strains to one 25 times greater. Need for added pantothenate was absolute in four cases, while in the other three suboptimal growth could occur without it. *Faecalis* strains were as a whole much less sensitive ; one organism grew normally without pantothenate, and was not inhibited by 2500 times the pantoyltaurine adequate for inhibition of Group A strains. It is relevant to later work that, in the streptococci of Table I, B, great variation occurred in resistance to pantoyltaurine without the organisms altering in their need for pantothenate, though nutritionally less exacting strains were, in a given group, among those least inhibited.

Prepared strains.—Development of resistant strains was attempted with four organisms, three of Group A and one of Group G. Resistant strains were obtained from three of these ; of Group A organisms, one whose growth was suboptimal in the present medium did not yield a markedly resistant strain after 16 subcultures. Resistance in the other organisms was evident after 8 subcultures, and the progress and technique of such training of one organism is illustrated in Table II. As the medium employed in training was not a normal bacteriological one, the effect of repeatedly subculturing each organism in it for 12 to 15 times was investigated ; the process was not found to alter their resistance to pantoyltaurine. When the final pantoyltaurine-trained organisms were compared with their original strains in growth from small inocula, they were found capable of growing in 10 to 50 times the highest concentration of pantoyltaurine which was tolerated by their parent strains (Table I, C). The rate and type of growth had not changed in training. The resistance of the derived strains was not transitory ; before each comparative test the trained organisms were subcultured twice on serum-broth-agar, and thus no temporary adaptation would be detected. In addition, the strain G_{PT}, derived from the Group G organism, was serially subcultured 25 times on serum-broth-agar without change in its resistance ; A_{2PT} was stable to 15 such subcultures, but A_{PT} was less stable, and after 15 sub-

TABLE I.—*Reactions of Streptococci to Sulphanilamide, Pantoyltaurine, and to Absence of Pantothenate.*

A. *Showing pantoyltaurine susceptibility to be independent of sulphanilamide resistance.*

Organism.	(Pantoyltaurine)/(pantothenate) ratio.			Growth without pantothenate.	Sulphanilamide (*M*).	
	Maximal for no inhibition.	Minimal for inhibition of 2 days.	Minimal for inhibition of 7 days.		Maximal for no inhibition.	Minimal for inhibition of 2 days.
Normal Group A haemolytic streptococci (6)	20	100	200	None	$2–4 \times 10^{-5}$	$1–2 \times 10^{-4}$
Sulphonamide resistant Group A streptococci — 3 strains	20–50	100	200–400	„	2×10^{-3}	$>2 \times 10^{-3}$
Sulphonamide resistant Group A streptococci — 8 strains	20	100	200	„	2×10^{-3}	$>2 \times 10^{-3}$

B. *Showing natural variation in pantoyltaurine susceptibility.*

Faecalis streptococci	II	500,000	..	..	Optimal	..	..
	3011	20,000–100,000	500,000	..	None	..	..
	III	20,000	100,000	..	„	..	..
	I	10,000	100,000	..	„	..	..
Viridans streptococci	1080	1,000	5,000	..	Suboptimal	..	..
	SU	200	..	5,000	None	..	..
	3165	100	..	5,000	Trace	..	..
	U	100	..	1,000	None	..	..
	3166	50–100	..	1,000	Trace	..	..
	E1	50	..	200	None	4×10^{-5}	2×10^{-4}
	E2	25–50	..	200	„	..	..
Group D strain		100	500–1,000	2,000	„	..	..
H69D strain		2,000–10,000	10,000	..	„	..	..

C. *Showing pantoyltaurine-training and its independence of sulphanilamide-susceptibility.*

Haemolytic streptococcus Group G	20	100	200	None	$2–4 \times 10^{-5}$	$1–2 \times 10^{-4}$
Strain G_{PT}	400–800	2,000–4,000	10,000	„	$2–4 \times 10^{-5}$	10^{-4}
Haemolytic streptococcus Group A (Richards)	20	100	200	„	2×10^{-5}	10^{-4}
Strain A_{PT}	200	1,000	2,000	„	2×10^{-5}	10^{-4}
Haemolytic streptococcus Group A	20	100	200	„	..	10^{-4}
Strain $A2_{PT}$	200	1,000	2,000–4,000	„	..	$1–2 \times 10^{-4}$

cultures was resistant to only twice the pantoyltaurine concentrations of the original strain.

Each strain was also compared with its parent organism in behaviour towards sulphanilamide. Little or no change in inhibition by sulphanilamide was found to have accompanied pantoyltaurine-training. On the other hand, G_{PT} was found to be less sensitive than G towards pantoyltauramide (McIlwain, 1942*c*) and salicylate (see below), whose actions are related to that of pantoyltaurine.

Nutritional properties of resistant organisms.

The three experimentally prepared pantoyltaurine-resistant streptococci retained a need for pantothenate, and did not vary greatly in their quantitative requirements of the compound. Neither the prepared strains, nor those of the naturally pantoyltaurine-resistant streptococci which needed pantothenate in growth, could utilize β-alanine in place of pantothenate ; a range of concentrations of $10^{-6}M$ to $10^{-3}M$ was tested. In this they differ markedly from resistant *C. diphtheriae* strains described below. In studying other possible mechanisms for this resistance, the observation

TABLE II.—*Process of Training a Group G Haemolytic Streptococcus to Grow in the Presence of High Concentrations of Pantoyltaurine.*

Subculture.	1st.		2nd.	3rd.		4th.	5th.		6th.		7th.		8th.	9th.		10th.		11th.	
Growth after (days):	1	4		1	3		1	5	1	3	1	3		1	3	1	4	1	4
Pantoyltaurine (*M*)—																			
4×10^{-6}	+	+		+	+		+	+											
2×10^{-5}	0	+ →	No change →	tr.	+ →	Little change * →	tr.	+	+	+									
5×10^{-5}	0	0		0	0		0	+ →	tr.	+	+	+							
10^{-4}	.	.	.	.	.	.	.	.	tr.	+ →	+	+							
2×10^{-4}	.	.	.	.	.	.	.	.	.	.	tr.	+* →	Little change	tr.	+				
5×10^{-4}	.	.	.	.	.	.	.	.	.	.	0	0		0	tr.* →	+	+		
10^{-3}	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	+ →	+	+
2×10^{-3}	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+
5×10^{-3}	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	tr.

Pantothenate throughout: $5 \times 10^{-8}M$. Growth: +, moderate or full; tr., trace; 0, none. Arrows indicate the main source of inocula for the succeeding subculture; in the cases marked with asterisks much-delayed growth from earlier subcultures was used in addition.

TABLE III.—*Correlation of Pantoyltaurine-resistance in Strains of C.* diphtheriae *with their Ability to Grow with β-alanine in Place of Pantothenate.*

Delay in growth on replacing pantothenate ($10^{-7}M$) by β-alanine ($10^{-5}M$) (days).	Strains requiring, for complete inhibition of visible growth, ratios of pantoyltaurine to pantothenate concentrations of—			
	1000 or less.	2000.	5000.	(Not inhibited by 5000.)
6	G1(m); 1248(g); 1217(g); 1318(g); 351(m)	1158(g); 1044(m); 907(g); 779(g)	..	..
2	..	..	G1156(m)	..
1	..	..	GW1(g)	1111(m); 351_{PT}(m)
0	..	..	..	GW1_{PT}(g); G1_{PT}(m); R_7(m); R_{20}(g); 1111_{PT}(m).

(m) = *mitis*, (g) = *gravis*. Further details of the prepared pantoyltaurine-resistant strains (G1_{PT}, etc.) are given in Table II, Part II.

of Woolley (1939) that a haemolytic streptococcus was capable of growing when pantothenate was replaced by pantoate was considered. Utilization of pantoate by the organism concerned (H 69 D ; I am indebted to Dr. Woolley for sending a culture) did not, however, take place in the media used in the present investigations, nor in various modifications of them except some which (like Woolley's medium) contained reduced iron. Under such conditions (with the further addition to the casein hydrolysate medium of threonine, 0·1 mg./ml. ; adermin, 5 μg./ml., and reduced iron, 1 mg./ml.) a Group D organism, a *viridans* and two *faecalis* strains which otherwise needed pantothenate were able to use pantoic acid in growth, though not as effectively as pantothenate. There was no consistent difference between pantoyltaurine-sensitive and -resistant strains in this respect.

Pantoyltaurine Susceptibilities of C. diphtheriae *Strains.*

Naturally-occurring variation in different strains.—Fourteen strains, 6 of *mitis* and 8 of *gravis* type, were examined. The most sensitive were inhibited by ratios of pantoyltaurine to pantothenate concentrations of 500, while others were unaffected by ratios of over 25,000. Marked differences were also found in the abilities of the organisms to grow with β-alanine in place of pantothenate. The variation in drug-resistance and in nutritional properties is summarized in Table III, when the two are seen to be correlated. All organisms which were inhibited by ratios of 2000 or less were unable to grow within six days in pantothenate-free media containing $10^{-5}M$ β-alanine, while all organisms growing without delay from that concentration of β-alanine were not inhibited by (pantoyltaurine)/(pantothenate) ratios of 5000 or more. A few strains showed intermediate properties. These results extend those found previously (McIlwain, 1942*b*), and are distinct from the present findings with streptococcal strains.

Preparation of pantoyltaurine-resistant strains.—Training experiments of the type carried out with streptococci were performed with four organisms : GW1 (*gravis*), 351, 1111, and G1 (all *mitis*). Marked increase of resistance was obtained in each case except that of 1111, which was initially only slightly sensitive to pantoyltaurine. The concentration of pantoyltaurine needed to inhibit the trained strain $G1_{PT}$ was 50 times that required to inhibit G1 in the presence of the same amount of pantothenate ; with 351_{PT} the ratio was 40 and with $GW1_{PT}$, 25 to 50.

Moreover, the pantoyltaurine-trained strains were changed in their nutritional properties. Although it was found that the organisms could be repeatedly subcultured in the present medium with $10^{-7}M$ pantothenate without losing their need for that compound, the pantoyltaurine-trained strains were less exacting than their parent organisms. Pantothenate ($10^{-7}M$) had been supplied throughout the training. The strains $G1_{PT}$ and $GW1_{PT}$ were capable of growing without delay when pantothenate was replaced by $10^{-5}M$ β-alanine, and 351_{PT} grew with a delay of one day. The change was especially marked with G1, in which case, as is indicated in Table III, the parent strain is completely exacting and strongly inhibited, while the derived $G1_{PT}$ is non-exacting and little inhibited.

Synthesis of pantothenate by derived strains.—Pantothenate is known to occur in some organisms which have grown in its absence ; such synthesis is clearly relevant to the action of pantoyltaurine, and the derived *C. diphtheriae* strains were examined in that respect. Strain $G1_{PT}$ was grown in 100 ml. batches of the casein hydrolysate medium containing $10^{-5}M$ β-alanine. After three days the organisms were separated and the fluid, sterilized by filtration, tested for pantothenate by its effect on growth of *Proteus morgani* and *Streptococcus haemolyticus* (McIlwain and Hawking, 1943). Absence of growth promoting or inhibiting properties in the filtrate showed it to

contain $<5 \times 10^{-9}M$ pantothenate. The bacteria were examined as follows, as much pantothenate is known to be bound in cells: organisms from a 100 ml. culture (dry weight, 55 mg.) were suspended in 1 ml. of phosphate buffer at pH 7·6, and allowed to autolyse for 48 hours at 37° with the addition of benzene. They were then diluted to 5 ml., autoclaved to sterilize and to remove benzene, and centrifugates assayed for pantothenate. They were found equivalent to $2 \times 10^{-7}M$ pantothenate both by *Str. haemolyticus* and *Pr. morgani*. A similarly-prepared autolysate from 351_{PT} contained $1–2 \times 10^{-7}M$ pantothenate.

Pantoyltaurine-resistance of Organisms Requiring Pantothenate in Growth.

Absence of pantoyltaurine destruction.—To the examples of natural resistance of this type in *Proteus morgani* (McIlwain, 1942*c*), some lactobacilli, a propionibacterium and *Leuconostoc mesenteroides* (Snell, 1941) have now been added the pantoyltaurine-trained haemolytic streptococci of the present investigation. Resistance in these cases cannot be by pantothenate synthesis, and the following experiment shows that it is not by destruction of pantoyltaurine. The strain G_{PT} was grown in the casein medium containing $10^{-3}M$ pantoyltaurine and $5 \times 10^{-8}M$ pantothenate. After maximal growth the culture was centrifuged and the fluid sterilized by filtration; dilutions from it were found to inhibit normal streptococci to the same extent as did similar dilutions of $10^{-3}M$ pantoyltaurine. Attempted growth of *Proteus morgani* in solutions containing the filtrate showed no increase in the pantothenate of the culture; growth in control tubes containing excess pantothenate as well as the filtrate showed it to be free from non-specific inhibitory compounds. A similar experiment in which *Pr. morgani* (N.C.T.C. No. 2818) was grown with pantoyltaurine showed an apparent reduction in the pantoyltaurine concentration to one half. In several experiments this reduction was no greater, but sometimes less; it is inadequate to account for the pantoyltaurine-resistance of the organisms, which are insensitive to over 1000 times the pantoyltaurine concentration by which Group A streptococci are inhibited. The resistant *C. diphtheriae* strain 351_{PT} was also shown not to destroy detectable amounts of pantoyltaurine when grown in its presence.

Synergism of pantoyltaurine with salicylate.—It was attempted to detect peculiarities in these organisms by the action of further inhibitors. The inhibitory ranges of several compounds with respect to *Pr. morgani* in the hydrolysed casein medium were determined, and concentrations which were (*a*) just without effect and (*b*) causing temporary inhibition, were examined also in the presence of $10^{-3}M$ pantoyltaurine; the basal medium contained $5 \times 10^{-8}M$ pantothenate. The compounds first tested were borax, sodium tellurate, arsenite, azide, iodate and cyanide; formaldehyde, urethane, veronal, salicylate, hexylresorcinol, acriflavine, methylene blue, quinine and penicillic acid (for which I am indebted to Prof. H. Raistrick). Of these only salicylate showed synergism with pantoyltaurine; 0·007 *M* salicylate alone caused little or no inhibition of *Pr. morgani*, but in the presence of $10^{-3}M$ pantoyltaurine, visible growth was completely inhibited for 7 days. Concentrations of 2×10^{-4} and $4 \times 10^{-5}M$ pantoyltaurine caused temporary inhibition. Of compounds structurally related to salicylate, benzoic acid and phenol were without synergistic effect with pantoyltaurine, but a small effect was found with anthranilate.

In studying the relation of this synergism to pantothenate, it was found that the inhibition of *Pr. morgani* by salicylate itself was antagonized by pantothenate; this has recently been reported also by Ivánovics (1942) to be the case with *Staphylococcus aureus*. The antagonism in growth of *Pr. morgani* occurred only in the case of inhibitions caused by limiting concentrations of salicylate; those which were completely

inhibitory (0·036*M*) were not antagonized by concentrations of pantothenate up to $10^{-3}M$. On the other hand, a mixture of pantoyltaurine ($10^{-3}M$) and salicylate (0·007*M*), which was completely inhibitory, was antagonized by $10^{-6}M$ pantothenate. The behaviour of *Pr. morgani* in the presence of this concentration of salicylate is thus similar to that of a moderately sensitive streptococcus or *C. diphtheriae* strain. Synergism of pantoyltaurine and salicylate was found to occur with both the normal and resistant streptococci G and G_{PT}, but larger quantities of both compounds were required to inhibit the latter organism.

DISCUSSION.

In these experiments two types of strains resistant to pantoyltaurine have been developed in bacteria. One, observed in *C. diphtheriae*, always showed increased ability to use β-alanine in place of pantothenate. The other, developed in *Str. haemolyticus*, remained exacting in respect to pantothenate. As experimental conditions in the two cases were similar, the changes which took place were characteristic not only of the drug (see below), but also of the organism. In each organism the change was to the type preponderating in naturally-occurring resistant strains of the same organism.

Pantoyltaurine-resistance in C. diphtheriae.—Typical resistant organisms, capable of growing from β-alanine and not requiring added pantothenate, were found to contain pantothenate, and thus differed from their drug-sensitive parent strains in synthesizing a compound which is known to be a specific antagonist to the drug. This is offered as a mechanism for their resistance. Comparable instances of the production of drug antagonists by resistant strains has previously been reported in some, but not all, cases of sulphonamide-fast organisms, and has recently been made more specific by the finding by Landy, Larkum, Oswald, and Streightoff (1943; references given to earlier literature) that sulphathiazole-fast staphylococci produced more *p*-aminobenzoate than did their parent strains. Of other mechanisms which have been suggested in explanation of various cases of drug resistance, some are shown above to be inapplicable to the present *C. diphtheriae* strains; thus resistance is not by destruction or precipitation of the inhibitor (cf. Neuschlosz, 1919). It is also improbable that it could be explained by permeability differences, for to supersede a metabolic explanation, such a hypothesis in the present case of inhibition by a metabolite analogue would require a "membrane" of greater specificity than the relation of enzyme to substrate.

Pantoyltaurine resistance in Str. haemolyticus.—In relation to the trained streptococcal strains, all three suggestions—production of antagonist, removal of inhibitor, and permeability differences—appear inapplicable. This is a type of change which has previously been referred to changes in the drug receptors. The simplest description in terms of receptors would be their increase in the pantoyltaurine-resistant organisms, and their blocking by salicylate. Reasons have been given (McIlwain, 1942*b*, *c*) for considering pantoyltaurine receptors to be essential enzymes which for growth must react with pantothenate. These may be inhibited by both pantoyltaurine and salicylate. Another biochemical mechanism consistent with the present findings would be the developing of alternative reactions, by the resistant organisms, to some of those processes which in normal organisms require pantothenate. A comparable instance in which this is known to occur is the temporary development by *Propionibacterium pentosaceum* of fluoride-insensitive glucose-dissimilating processes when grown in the presence of fluoride, which inhibits the reaction in normal strains (Wiggert and Werkman, 1939). Such a change, and also the change in the trained *C. diphtheriae* strains, cannot be referred to a change in receptors without implying that the receptors

are complicated systems involving to an indefinite extent the general metabolism of the organism. It would appear better to maintain the restriction of the term "receptor" to the immediate point of action of the inhibitor, from which it follows that resistance may be caused by changes in systems whose connection with a receptor can be exp[illegible]ed only in metabolic terms. Instances are known of drug-resistant strains whic[illegible]iffer metabolically from their parent organisms (Marks, 1910; Revis, 1912). Kohn and Harris (1942) also found a nutritional difference to develop with drug resistance; in that case the organisms became nutritionally more exacting.

Sulphanilamide- and pantoyltaurine-resistance.—The results have demonstrated that a new chemotherapeutic agent (A′) related in structure to a metabolite (M′) as is a known agent (A) to a metabolite (M) does not necessarily lead to the formation of strains showing cross-resistance with (A). This is a further indication of the biochemical specificity of chemotherapeutic agents, which was previously emphasized (McIlwain, 1942*d*) from experiments on their relationship to their natural antagonists. If, in the *C. diphtheriae* strains, resistance is due to pantothenate synthesis, no change in sulphanilamide-resistance would be expected, as pantothenate is known not to affect sulphanilamide-inhibition. Similar absence of cross-resistance in streptococcal strains indicates that although a detailed mechanism cannot yet be given for their pantoyltaurine resistance, it is one relatively specific to pantoyltaurine and unconnected with *p*-aminobenzoate.

SUMMARY.

The method of designing chemotherapeutic agents which is exemplified by pantoyltaurine does not necessarily lead to cross-resistance with the inhibitor used as model; sulphonamide-resistant streptococci were of normal sensitivity to pantoyltaurine, and pantoyltaurine-fast organisms were as susceptible to sulphanilamide as were normal strains.

The varying resistance to pantoyltaurine, of different natural and experimentally prepared strains of *C. diphtheriae*, was correlated with their ability to grow with β-alanine in place of pantothenate. Resistant strains synthesized pantothenate in growth, and as pantothenate antagonizes pantoyltaurine, this synthesis is considered to explain their resistance.

Resistance of *Str. haemolyticus* to pantoyltaurine is not by this process, as many of the naturally resistant, and all the experimentally produced resistant strains, still needed pantothenate in growth. The pantoyltaurine-fast streptococcal strains, and other organisms insensitive to pantoyltaurine alone, were made susceptible to it by the further addition of salicylate. Inhibition by salicylate is related to pantothenate, which in some cases antagonizes it; it is suggested that the pantoyltaurine-resistant streptococci differ from their parent strains in possessing metabolic processes alternative to those susceptible to pantoyltaurine, and that these are susceptible to inhibition by salicylate.

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NUTRITIONAL STUDIES OF BACTERIAL VARIATION. II. THE DERIVATION OF DRUG-RESISTANT STRAINS IN THE ABSENCE OF ANY INHIBITOR.

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IN seeking to understand and possibly to control the development, in micro-organisms, of strains which are resistant to chemotherapeutic agents, it is of first importance to know whether such development is conditional on the presence of the inhibitors, or whether it can occur without them. Resistant strains of organisms derived by the use of certain inhibitors are known to be resistant to other agents, but this is believed to be due to a common action on the part of such groups of inhibitors, and is distinct from the present study. A comparison was reported in Part I, of organisms which were resistant to pantoyltaurine when first isolated, with others which had been derived experimentally by growth in the presence of pantoyltaurine, and this comparison suggested there to be no fundamental difference between the two types of resistant organisms. The mechanisms of their resistance could be suggested, and in the artificially prepared strains of *C. diphtheriae* appeared to be the same as that of strains which were naturally resistant. As it was unlikely that the naturally resistant strains had been in contact with pantoyltaurine, they had presumably arisen through other circumstances. The present paper describes attempts to reproduce, under experimental control and without the use of an inhibitor, a similar variation in the resistance of strains to pantoyltaurine.

The method employed was suggested by the following considerations. (*a*) Resistance to pantoyltaurine in *C. diphtheriae* strains was correlated with the ability of the strains to utilize β-alanine in place of pantothenate in growth. (*b*) Strains of *Staphylococcus aureus* and of *Bacterium typhosum* which had been nutritionally trained to be independent of added α-aminocarboxylic acids in growth, were more resistant to the inhibitory action of α-aminosulphonic acids than were their parent strains (McIlwain, 1941). The aminosulphonic acids appeared to be inhibitory by their interference with aminocarboxylic acid metabolism. This itself probably represents the production of resistant strains in the absence of inhibitors (McIlwain, 1942), but as the strains had been derived four years prior to tests with inhibitors, and the aminosulphonic acids

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could not be considered chemotherapeutic agents, the relevance of the results to chemotherapy might be questioned. Direct nutritional training of the present *C. diphtheriae* strains to grow with β-alanine in place of pantothenate has therefore been attempted, and the pantoyltaurine resistance of the resulting organisms investigated.

EXPERIMENTAL.

The media and techniques were those employed in Part I. Each initial strain was repeatedly subcultured from single colonies before the commencement of training experiments.

Training to utilize β-alanine in place of pantothenate.

By use of added β-alanine.—The training was first attempted with four of the strains of *C. diphtheriae* previously investigated, namely G1, 351, 1111 (all *mitis*) and GW1 (*gravis*). When pantothenate-free media containing β-alanine were inoculated with the organisms, growth was in all cases suboptimal. From the normal small inocula of *c.* 100 organisms/ml., and in the presence of $10^{-5}M$ β-alanine, GW1 and 1111 grew after a delay of 1 day, while G1 and 351 did not grow within 7 days. With large inocula all organisms grew, though again their rate of growth varied. Training was therefore carried out by the general procedure described in Part I, with varying inocula, and with varying concentrations of β-alanine. The organisms were subcultured successively from those tubes containing the lowest concentrations of β-alanine, in which growth had occurred from the smallest inocula. The progress of training G1 is given in Table I, in which the results of growth from small inocula (*c.* 1000 organisms in 10 ml. of medium) are recorded. At the same time, a similar series of tubes with falling concentrations of β-alanine were inoculated with *c.* 10^6 organisms in 10 ml. of medium. Successive subcultures were made from any tubes in which delayed growth occurred from limiting concentrations of β-alanine; the arrows of the Table thus indicate one, but not the only, source of inocula. The tubes with larger inocula necessarily formed the sole source of inocula in the first two subcultures, but after this stage did not illustrate well the progress of the training. After 12 subcultures, the derived G1β-al. grew more quickly from $10^{-7}M$ β-alanine than did the original strain with 100 times this quantity. There was little or no difference between the ability of the trained strain to grow with optimal β-alanine and with optimal pantothenate, though the latter remained the more effective growth factor as β-alanine was required at about $2 \times 10^{-6}M$ for rapid growth, and pantothenate at $5 \times 10^{-8}M$. A comparable difference exists in the optimal β-alanine and pantothenate requirements of naturally less-exacting *C. diphtheriae* strains.

TABLE I.—*Progress of Training an Exacting* C. diphtheriae *Strain (G1) to Grow with β-alanine in place of Pantothenate.*

Subculture:	1st, 2nd.	3rd.	4th.	5th.	6th.	7th, 8th.	9th.	10th.	11th, 12th.
Growth after (days):	5 · 1	5	1 4		1 3		1 2	1 2 6	
β-alanine (*M*)—									
10^{-5}	0 0	+	0 +	No change	tr. +	No change	tr. +		No change
2×10^{-6}	0 0	tr. →	0 + →	→	0 +	→	0 +	+ +	
4×10^{-7}	0 0	0	0 0		0 tr. →		0 tr. →	0 + +	
2×10^{-7}	. .	.	. .		. .		. .	0 0 tr. →	
10^{-7}	. .	.	. .		. .		. .		

The results of similarly training the other three organisms are given in Table II. The findings with 351 are comparable with those with G1, as the original strain is

exacting, while 351$_{\beta\text{-al.}}$ was trained to grow in 1 day from $2 \times 10^{-6}M$ β-alanine. Strain GW1 initially grew from $5 \times 10^{-6}M$ β-alanine in 2 days, but was trained to grow from $10^{-6}M$ in 1 day; 1111$_{\beta\text{-al.}}$ finally grew promptly without added β-alanine. The derived strains were in all cases examined after subculture under the same conditions as the original strains, that is, on serum-broth-agar. In addition, G1$_{\beta\text{-al.}}$ and 351$_{\beta\text{-al.}}$ were examined after 16 serial subcultures on that medium, and found unchanged in their ability to grow with β-alanine in place of pantothenate.

Training without addition of β-alanine.—It was previously found (Part I, p. 208) that *C. diphtheriae* strains, including those now being described, did not change in nutritional properties when serially subcultured many times in the present medium containing $10^{-7}M$ pantothenate. They have, however, been found to react to subculturing in the presence of markedly changed concentrations of the compound.

Training of G1 and GW1 to be independent of added pantothenate was attempted by growing them from inocula of *c.* 10^6 cells in solutions containing 10^{-7} to $10^{-9}M$ pantothenate, and successively subculturing those organisms which had appeared in the tubes containing least pantothenate. During this process, both organisms commenced to grow more quickly without added pantothenate, and the properties of the G1$_{-P}$ and GW1$_{-P}$ resulting after 11 subcultures are given in Table II. They thus appear to have changed in the same sense as G1$_{\beta\text{-al.}}$ and GW1$_{\beta\text{-al.}}$, presumably by making use of the β-alanine of the basal medium.

Derivation of strains more exacting in their pantothenate requirements.

The properties of an organism resulting from growth in mixtures of pantothenate and β-alanine can thus be altered by altering the relative concentrations of the two compounds. The effect of altering the mixture so that it contained much more pantothenate was next examined. Each of the organisms G1, GW1, 351 and 1111 were subcultured in the presence of $10^{-4}M$ pantothenate, which is about 2000 times the minimal concentration for full growth. After 15 successive cultures, the resulting strains 1111$_P$ and GW1$_P$ were found to have become more exacting. Growth of 1111$_P$ from small inocula without added pantothenate was delayed; GW1 normally gave little or no growth without pantothenate, but GW1$_P$ now required much more β-alanine for growth in the absence of pantothenate. Strain 1111$_P$ was also feebler than the original strain in its response to β-alanine. Strains 351 and G1, which were already among the most exacting of the normal organisms examined, were not changed by this process. Strains GW1$_P$ and 1111$_P$ remained more exacting than their parent strains after 16 successive subcultures on serum broth agar; no change was detected in 1111$_P$ but GW1$_P$ showed increased ability to grow from β-alanine.

Behaviour of the derived strains towards pantoyltaurine.

This was examined by growth from small inocula in the presence of $10^{-7}M$ pantothenate and varying concentrations of pantoyltaurine. In all cases the derived strains were compared with the original organisms with the same batches of reagents and by subcultures from similarly-grown inocula.

One or more of the derived strains from each of the four parent organisms were found to have changed in their behaviour towards pantoyltaurine. The changes are summarized in Table II, and were always in a sense which correlated increased nutritional demands with increased drug sensitivity. Thus the strains G1$_{\beta\text{-al.}}$ and G1$_{-P}$, derived from an organism of absolute pantothenate-requirement but which were now trained to use β-alanine in place of that compound, required 10 to 50 times more pantoyltaurine for their inhibition than did their parent strain. With 351$_{\beta\text{-al.}}$ and

351 this factor was 10; GW1β-al. and GW1$_{-P}$ bore a similar relationship to GW1, which initially showed some pantoyltaurine resistance; the derived strains could not now be completely inhibited by extremely high (pantoyltaurine)/(pantothenate) ratios. Strain 1111 was already highly resistant, and little change could be detected after β-alanine training. On the other hand, 1111$_P$, which was much more exacting than 1111, showed much greater susceptibility to pantoyltaurine. The strain GW1$_P$ differed in the same sense from GW1. Strains G1$_P$ and 351$_P$ had not changed in either nutritional properties or pantoyltaurine-susceptibility from their parent organisms, which were already among the most exacting and sensitive of the organisms examined.

TABLE II.—*Summary of Properties of Trained Organisms.*

Strain.	Minimal β-alanine (*M*) for growth in—		Ratio of pantoyltaurine to pantothenate concentrations.	
	1–2 days.	3–4 days.	Maximal for no inhibition.	Minimal for inhibition of 4 days.
G1 and G1$_P$	(None with 10^{-5})	(None with 10^{-5})	50–100	200–500
G1$_{-P}$	(Trace with 10^{-5})	2×10^{-6}	200	2,000
G1β-al. and G1$_{PT}$	10^{-6}	10^{-7}	2,500	10,000
GW1	5×10^{-6}	2×10^{-6}–4×10^{-7}	500	4,000–5,000
GW1$_P$	(None with 10^{-5})	(Trace with 10^{-5})	200	800–1,000
GW1$_{-P}$	4×10^{-7}	No addition	2,000	>20,000
GW1β-al.	10^{-6}	,,	4,000	>20,000
GW1$_{PT}$	4×10^{-7}	,,	2,000–4,000	>20,000
351 and 351$_P$	(None with 10^{-5})	10^{-5}	100–200	1,000–2,000
351β-al.	2×10^{-6}	4–8×10^{-7}	1,000–2,000	20,000
351$_{PT}$	10^{-5}	4–8×10^{-6}	10,000	100,000
1111	2×10^{-6}	No addition	200–500	>100,000
1111$_P$	10^{-5}	2×10^{-6}	100–200	2,000
1111β-al. and 1111$_{PT}$	No addition	No addition	>100,000	>100,000

In Table II are shown the characteristics of the resistant strains derived as described in Part I, by use of pantoyltaurine, as well as those which have now been prepared with β-alanine or by removal of pantothenate. Although none of the processes has been taken to a definite end-point, and the resulting organisms are not expected to be the same, differences are shown which indicate that the processes are not entirely comparable. Thus, following the general tendency previously described, GW1$_{-P}$ and GW1β-al. have both greater pantoyltaurine resistance and greater ability to use β-alanine than has GW1; but GW1$_{-P}$ uses β-alanine more effectively than GW1β-al. and is the *more* sensitive to pantoyltaurine. Strains 351β-al. and 351$_{PT}$ are similarly related to 351 but, between themselves, again show an inverted relationship comparable to that between GW1$_{-P}$ and GW1β-al. The deviations from complete correlation between β-alanine usage and pantoyltaurine resistance are, however, small in these cases and were not found in others; they are comparable to the spread in these properties shown in Table III, Part I, when GW1 and 1111 were found similar in respect to their β-alanine usage but different in their resistance. Resistance is therefore affected to some extent by conditions other than β-alanine usage (*cf.* the results with streptococci, Part I), or β-alanine usage by conditions which do not affect pantoyltaurine resistance.

Other properties of C. diphtheriae *strains.*

Stability.—The pantoyltaurine-resistance of G1β-al., 351β-al., and 1111$_P$, as well as their ability to use β-alanine, was found to be unaffected by 12 subcultures on normal laboratory media. The starch-fermenting characters of the derived strains placed them in the same types as their parent organisms. This was of diagnostic value, but

could not have been anticipated, as drug resistance has previously been found to be associated with changes in such properties, and pantothenate is known to be connected with carbohydrate metabolism. The present findings, however, emphasize that, in the strains investigated, pantothenate-requirement is independent of the Type of *C. diphtheriae*.

Synthesis of pantothenate by trained strains.—By the methods described in Part I, which had demonstrated $G1_{PT}$ and 351_{PT} to synthesize pantothenate, $G1_{\beta\text{-al.}}$ also was found to excrete little or no pantothenate to the pantothenate-free medium containing $10^{-5}M$ β-alanine, but to yield an autolysate containing $3 \times 10^{-7}M$ pantothenate. Strain 351_{PT}, under similar conditions, excreted pantothenate, yielding a culture fluid containing $2 \times 10^{-8}M$ pantothenate after 3 days; the cells on autoclaving in 5 ml. of buffer yielded $2 \times 10^{-7}M$, and on autolysis, $5 \times 10^{-7}M$ pantothenate.

Assimilation of pantothenate by parent strains.—The behaviour of the trained G1 strains was compared with that of G1. The parent organism was grown in batches of 100 ml. of the casein hydrolysate medium without added β-alanine, but with (*a*), $10^{-6}M$ and (*b*) $10^{-7}M$ pantothenate. After 3 days the culture fluids contained $<3 \times 10^{-9}M$ pantothenate, but the cells from (*a*) yielded an autolysate (5 ml.) containing $1{\cdot}5 \times 10^{-7}M$ pantothenate, and from (*b*) one containing $7 \times 10^{-8}M$. Thus the organism, when grown in pantothenate even in excess of its minimal requirements, removed the great majority of it from the medium. The quantity extracted from its cells is comparable to that extracted by the same procedure from cells of the trained organisms growing in the absence of added pantothenate. Retention of pantothenate by the cells of the resistant organisms is thus not evidence for their resistance being due to permeability differences.

DISCUSSION.

On the basis of a hypothetical mechanism for pantoyltaurine-resistance in *C. diphtheriae* strains, drug-fast and drug-sensitive organisms have thus been derived experimentally by processes which employed neither the drug in question nor any related inhibitor. It is thus probable that the correlation, which was found in Part I, between the pantoyltaurine resistance of *C. diphtheriae* strains, and their ability to grow with β-alanine in place of pantoyltaurine, was not accidental. The correlation, in conjunction with the present experiments and the knowledge that pantothenate antagonizes the inhibition of pantoyltaurine, make it highly probable that the ability of a strain to synthesize pantothenate conditions its resistance to pantoyltaurine.

The present production of strains of varying drug-resistance by nutritional manipulation provides an indication of how such variations may arise naturally, and may be of epidemiological significance. Although the normal or laboratory environments of organisms rarely contain drugs unless these are deliberately added, they contain varying amounts of essential nutrients. The general knowledge that these will change in concentration during growth has been shown to be related to drug resistance by the demonstration that pantothenate is removed from solution during growth of *C. diphtheriae*, which thus itself tends to create a condition under which resistant strains are formed.

The results of both β-alanine and pantoyltaurine training are in agreement with a very general observation: that in changing in a new environment, an organism does so in a manner which renders it more suited to live under the new conditions. The changes following lack of pantothenate and presence of pantoyltaurine are similar; the observation that both conditions lead to strains using β-alanine in place of pantothenate gives a mechanism for the drug-resistance of the strains but not for their

origin. The processes by which variant strains of different organisms arise has, however, been studied in relation to changes in metabolic behaviour, and it is to such behaviour that the present results refer drug-resistance. Thus one hypothesis in the present instance would be that, as in the case of the lactose-fermenting ability of certain colon bacilli (Lewis, 1934), a small proportion of organisms in the *C. diphtheriae* strains is capable of pantothenate synthesis and that training selects this fraction. Yorke, Murgatroyd and Hawking (1931) argued against this view in relation to the resistance of trypanosomes to tryparsamide, and drug-resistance is commonly spoken of as induced or provoked by the drug, which would imply a more intimate relationship between the drug and development of resistance. The present results of β-alanine-training show, however, that this is not always an exclusive relationship. In the present work the following observations are relevant to the origin of the resistant strains : the minor differences in properties in some organisms between the β-alanine- and pantoyltaurine-trained strains, the changes in pantothenate-rich media, and the apparently gradual nature of the training. These would suggest either the existence of a very wide series of natural variants of any one organism with respect to one characteristic, or an influence, other than or as well as selection, on the part of the agent used in training.

SUMMARY.

Pantoyltaurine-resistant strains of *C. diphtheriae* have been prepared without the use of the drug or other inhibitor, by serial subculture (*a*) in the presence of much β-alanine and absence of pantothenate, and (*b*) in the presence of little β-alanine, but falling concentrations of pantothenate. Pantoyltaurine-sensitive strains have been obtained by repeated subculture in the presence of high concentrations of pantothenate. The derived resistant strains were stable to repeated subculture in normal laboratory media. The presence of the drug is thus not essential to the development of drug-resistant strains, and the natural variation in resistance of organisms not known to have been in contact with a drug may receive explanation in nutritional terms.

The characters of the resistance which arose in different organisms to pantoyltaurine depended mainly upon the type of organism, but to a lesser extent upon the means by which it was brought about. The bearing of this on the genesis of the derived strains is discussed.

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CRITERIA OF LIFE AND DEATH IN HUMAN PUS CELLS.

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In the study of cells it may be important to distinguish the living from the dead and the healthy from the moribund. The task presents great difficulties. Stained preparations supply little evidence of the state of the cells at the time of staining, and even the appearance of disintegration may be misleading, as, for example, in the case of the eosinophil leucocyte, which often extends itself into a slender thread, the granules lying like a chain of beads at a considerable distance from the nucleus. The intervening cytoplasm does not take colour from Leishman's stain, so that the cell stained in this way appears as a chain of scattered eosinophilic granules unconnected with the remote distorted pale blue nuclear lobes. The filiform elongation is a reversible process, and such a cell may subsequently round up. A good photograph of filiform eosinophil cells is contained in an interesting paper by Woodruff and others (1942, fig. 4), but the appearance is misinterpreted (p. 320) as indicating rupture of the cells.

Examples of this sort could be multiplied from the literature, and lack of precise knowledge of the behaviour and appearance of leucocytes renders almost valueless such distribution studies as those of Andrewes (1910) and Bruce (1894), and may have prejudiced our present conception of leucopoiesis.

Granular leucocytes provide evidences of viability which render easier than in non-motile cells the separation of the appearances of life from those of death. Even so the difficulties of interpretation are not negligible, and unfortunately the criteria of life and death accepted by different workers have been arbitrary and conflicting. The criterion has usually been the presence or absence of one or more of the following phenomena : (a) *phagocytosis*, (b) *pseudopodial activity*, (c) *granule motility*.

(a) *Phagocytosis* has been the most used, partly because its result can be detected in stained films and the painstaking scrutiny of the fresh preparation thereby avoided. Phagocytosis is, indeed, unimpeachable evidence of the living state of pus cells, but its absence is far from being evidence of their death or moribundity. Many reversible activities, some of which may involve the death of bacteria, proceed in the absence of phagocytosis.

(b) *Pseudopodial activity*.—If in fresh material the medium surrounding the cells be in motion—due to capillary pressure, evaporation, or other cause—pseudopodia may be passively evoked from leucocytes by such external movement, and would indicate life only if it were established that they could not be similarly educed from the dead cell. In a still medium pseudopodial activity is evidence of cell life, but as with phagocytosis, its absence is not evidence of death or moribundity.

(c) *Granule motility*.—Many (e.g. Downey, 1928) have interpreted the motility

of the cytoplasmic refractile granules as evidence of life, and its total cessation as an indication of death of the cell with irreversible gelation of the cytoplasm. Others, on the contrary, consider that granule motility is not seen except in dead (Sherrington, 1894 ; Jeanneret and Fischer, 1941) or moribund cells.

To sum up, one may say that phagocytosis and pseudopodial activity are inadequate indices in that their absence does not necessarily connote cell morbidity or death, and that the significance of granule motility has not been established. The problem was one which continually confronted me during certain studies on human pus cells, and the remarks which follow are based on observations extending over six years.

METHOD.

The death of a cell is a matter of fact and not of arbitrary definition. It may, of course, occur at any stage of development and during any phase of activity, and indeed pus cells because of their military functions commonly die in any stage. An accurate interpretation of the different appearances of pus cells was sought in a painstaking study of the *total history of the cell* from the first emergence of cytoplasmic refractile granules to disintegration of the cell into an amorphous material. It was hoped that such a study might provide evidence for a decision at what point in the history death had occurred, and how it might be recognized. Parallel studies on stained preparations of all material were made in order to correlate stained appearances with the phases of the living cell.

RESULTS.

It soon became apparent that living pus cells exhibit three types of changes :

(1) Reversible changes or *activities*.
(2) Irreversible changes representing *maturation*.
(3) Irreversible changes representing *degeneration*.

Fig. 1 shows some of the appearances of the unstained neutrophil in the three categories. The *activities* illustrated are :

a. Amoeboid pseudopodial activity.
b. Filopodial activity.
c. The ejection of pus-cell corpuscles.
d. Filiform elongation of the whole cell with or without filiform elongation of the nucleus.

All these *activities* are reversible, but when numerous pus cells exhibit the filiform change and take part in the formation of *ameobocyte tissue* (*paraplastic tissue*, "*web formation*")—as commonly happens in sputum—it is unusual for them to revert to the spherical shape. Cells dying during phases of activity pass through degenerative forms, but these together with some other appearances have been omitted from Fig. 1 for the sake of simplicity.

Maturation chiefly shows itself in increase in the amount of cytoplasm and the number of granules, and decrease in size and subsequent lobulation of the nucleus. A cell at any stage of maturation may exhibit any of the *activities*, and the degree of its nuclear lobulation will remain unchanged or progress to a higher degree of division, but will never return to a simpler nuclear configuration.

Degeneration forms are those which, being irreversibly derived from other forms, never themselves show any *activity*, and always progress to disintegration.

Fig. 2 shows the cells in Fig. 1 as stained with Leishman's stain, except that at the level of the first nuclear division the eosinophil is substituted for the neutrophil.

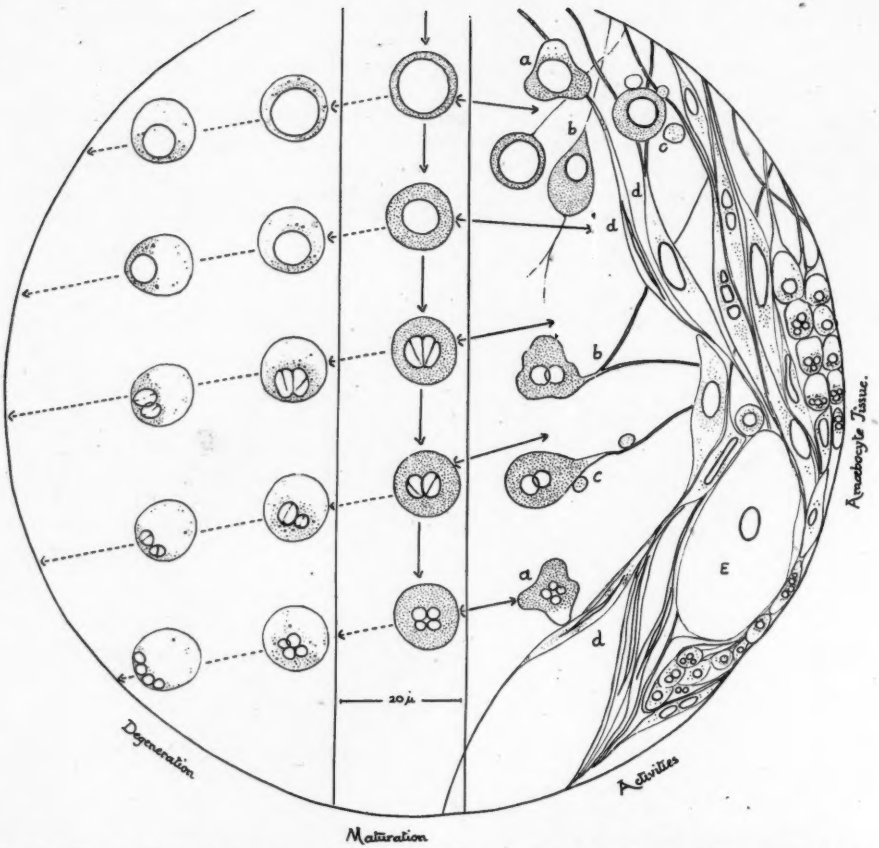

FIG. 1.—Various phases of the neutrophil pus cell. Every cell shown is a neutrophil except E, which is a pavement epithelial cell. Of the *activities* shown, (*a*) is amœboid movement, (*b*) filopodial activity, (*c*) production of pus cell corpuscles, (*d*) filiform elongation of the whole cell with or without filiform elongation of the nucleus.

To the right of the diagram dead and moribund neutrophils can be seen caught up in the meshes of the neutrophil amœbocyte tissue.

It should be noted that in purulent material the granules and cytoplasm of the neutrophil do not stain.

We must now attempt to show at what point death occurs, and to find evidence to corroborate the conclusions so far reached.

Significance of Granule Motility.

Examination of fresh material casts some light on the divergent views which are held on the subject of the motility of the cytoplasmic refractile granules. The subject may be examined under four heads as to the presence and absence of motility in live and dead cells.

(1) *Live cells can show granule motility.* This is proved by frequent observation of granule motility in cells showing various *activities* such as amœboid movement.

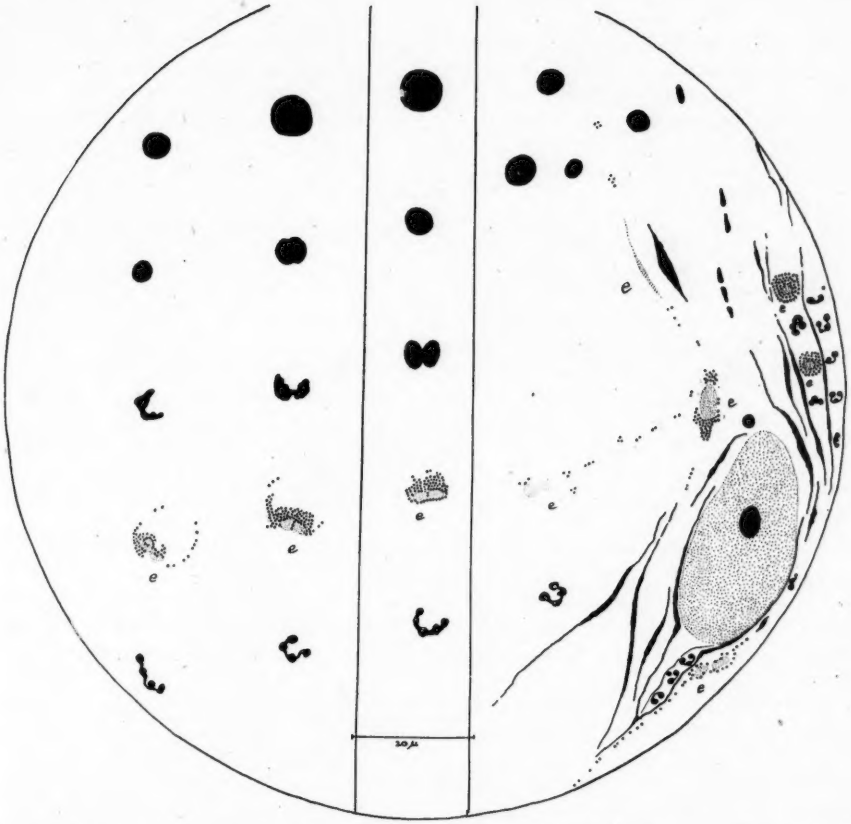

FIG. 2.—Represents fig. 1 as it might appear if the cells had been stained with Leishman-Wright stain, except that where the cells are marked "e" the neutrophils have been replaced by eosinophils. The granules of the neutrophils are not shown because they seldom stain in purulent material. Note the filiform nuclei of the cells on the right of the diagram, and the apparently dislocated granules of the extended eosinophils.

Black represents plum colour; coarse stippling, scarlet (eosinophil granules); fine stippling, pale blue.

(2) *Live cells can show complete absence of granule motility.* Cells whose granules are motionless may subsequently indulge in various *activities*. In elucidating this point one must beware of a trap in that cells have a phase in which their membrane becomes semi-opaque, like ground-glass, and it is difficult then to be sure what is the state of the granules. The surface looks granular and motionless, but it must not be concluded that the granules are stationary. Sometimes a small portion of the membrane will momentarily clarify, revealing a glimpse of turbulent granules. To confirm conclusion (2) the granules must be *seen* to be still.

In fact in a live cell the granules may all cease and resume motility many times. They may also be induced to do so artificially by appropriate manipulation.

(3) *The granules of dead cells can be motionless.*

(4) *Can dead cells show granule "motility"?* In the degeneration forms of cells dying slowly, fewer and fewer granules remain motile, the rest clumping irregularly in the cytoplasm or dropping in a cluster around the eccentric shrunken nucleus. When the last granule has come to rest motility is not resumed, and no further incident occurs except the more or less gradual disintegration of the cell. Let us here suggest tentatively that at this point—the final immobilization of all the granules of a degenerate cell—the cell dies, and that dead cells show no granule motility. We may then seek other evidence of the truth or falsity of the supposition.

Effect of Methylene Blue.

If 1 per cent. watery methylene blue be added to fresh material, three appearances will be noticed in pus cells :

(1) Some stain immediately, the nucleus intensely, and the cytoplasm to the same degree as the medium. These are those we have already posulated as dead—i.e. with non-motile granules and shrunken eccentric nuclei.

(2) Others show varying degrees of nuclear staining, the cytoplasm and most of the granules remaining clear and unstained against the blue matrix. These are those designated degeneration forms, and they show granule motility.

(3) Others again show no coloration at all. These are those we have on other grounds considered the healthier cells.

The appearances described are not to be explained on the assumption that the membrane of the living cells offers a resistance to the penetration of the stain which that of the dead cell does not. In such a case the nucleus could not become stained before the cytoplasm. Methylene blue is an oxidizing agent, the reduced product being colourless (the "leucobase") and capable of reoxidation, and it seems most probable that in the living cell the methylene blue is immediately reduced by the chemical activities of the living cytoplasm. The reaction depends on the integrity of a rather complex enzyme system. It is impossible to say if the nucleus colours before the cytoplasm because it has a more readily impaired reducing system, or more probably because it has none, and less intense activity of the degenerate cell allows the leucobase to reach the nucleus, where it regains its coloured form. Cells may remain for many days in a condition in which the nucleus is more or less deeply stained and the cytoplasm is colourless and refractile, before finally allowing the methylene blue to tint the cytoplasm. This final act never occurs before the immobilization of the granules. A solitary dancing granule is always in a clear colourless cytoplasm. The power to reduce methylene blue is lost at the moment, which on other grounds we have tentatively suggested is the moment of death. Evidence from the behaviour of the pus cell towards methylene blue therefore seems to support the evidence from simple study of the living cell as to the appearances of death, degeneration, and health in pus cells.

The longevity of pus cells appears to have been often under-estimated. It is worth recording that cells in sputum often live for as much as nine days at room temperature.

SUMMARY.

Various criteria of the vitality of leucocytes are criticized. A study of the total life-history of pus cells is correlated with a study of their action on methylene blue with the following conclusions :

Changes in pus cells are either reversible (i.e. *activities*) or irreversible.

Irreversible changes due to *maturation* do not interfere with the power to indulge

in *activities*. Irreversible changes due to *degeneration* are accompanied by loss of the power to perform *activities*.

Some of the appearances in the three categories and the misleading possibilities of stained preparations are illustrated.

Certain phenomena associated with cell-death are described.

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INFLUENCE OF CROSS-SUCKLING ON THE INCIDENCE OF MAMMARY CANCER IN HIGH AND LOW-CANCER STRAINS OF MICE.

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THE predominance of the maternal influence in the apparent hereditary transmission of the tendency to develop cancer of the breast in certain pure line strains of mice was established by reciprocal crosses between high and low cancer strains. When the female of a high-cancer line is mated with a male of a low-cancer line, the females of the progeny give rise to approximately the same percentage of mammary cancer as the pure high-cancer line, whereas the hybrids of a low-cancer line female and a high-cancer line male are practically exempt from mammary cancer. These facts have been well established by the staff of the Roscoe B. Jackson Memorial Laboratory (1933), by Korteweg (1934), and Murray and Little (1935).

These workers attributed the maternal influence to an extrachromosomal factor which could not be specified. Bittner (1936, 1937*a*, *b*, *c*, 1939*a*, *b*, 1940*a*, *b*, *c*) discovered that the extrachromosomal factor is conveyed in the mothers' milk. This discovery has been confirmed by many investigators (Andervont and McEleney, 1939*a*; Bagg, 1939; Murray, 1941; Korteweg, 1936; and by Andervont, 1940).

The present experiments were undertaken with strains of mice not hitherto used in experiments on the milk factor in order to investigate the general validity of the results already described.

METHODS.

Mice of RIII (Dobrowolskaja-Zawadzkaja) high-cancer strain and of "S" low-cancer strain were used in the present experiments. Strains RIII and "S" have undergone 25 and 19 generations of inbreeding respectively, since they were obtained from other laboratories. Strain RIII females are highly susceptible to the develop-

ment of mammary cancer; the incidence of tumours observed in this Laboratory is 83 per cent. at an average age of 8½ months. Strain "S" females are very resistant, giving an incidence of mammary cancer of less than 1 per cent. at an average age of 16 months (Table I).

TABLE I.—*Tumour Incidence in RIII High-Breast-Cancer Strain of Mice and "S" Low-Breast-Cancer Strain of Mice.*

Strain of mice.	Tumour incidence.	Average tumour age.	Average age of death without tumour.
RIII	83%	8·5 months	18 months
"S"	Less than 1%	19 ,,	22 ,,

The experiment was begun during June, 1940, by brother-to-sister matings in both strains of mice. Pregnant mice derived from these matings were examined twice daily at 9 o'clock a.m. and at 5 o'clock p.m. to find out whether they had littered. As the RIII young were born, they were transferred to a foster mother of the strain "S," and as the young "S" strain mice were born they were transferred to the RIII foster mother. After the mice were weaned, females of both strains were mated to their brothers and allowed to bear and bring up litters in the normal way.

The mice were kept under similar conditions and on a diet of Purina-chow cubes with an unlimited supply of water.

RESULTS.

The results are presented in Table II. This shows that foster nursing of young RIII females by females of strain "S" decreased the incidence of mammary cancer in RIII females from 83 per cent. to 6·6 per cent.—2 females out of 30 developed mammary cancer.

TABLE II.—*Tumour Incidence in Low-Breast-Cancer Strain of Mice Foster Nursed by High-Breast-Cancer Strain of Mice, and High-Breast-Cancer Strain of Mice Foster Nursed by Low-Breast-Cancer Strain of Mice.*

Strain used for foster nursing.	Foster nursed by—	Number of mice foster nursed.	Number of mice which developed breast cancer.	Number of mice which died free of cancer.	Tumour incidence.	Average tumour age.	Average life of mice free of cancer.
"S"	RIII	43	8	16 (before reaching tumour age); 19 (after reaching tumour age)	8 out of 27, i.e. 29·6%	15·2 months	22·3 months
RIII	"S"	35	2	5 (before reaching tumour age); 28 (after reaching tumour age)	2 out of 30, i.e. 6·6%	9·3 months	20·7 months

The results confirm the findings of Bittner and other workers, and demonstrate

whether RIII strain females fostered by low-cancer strain " S " mothers develop mammary cancer or not is dependent upon the time at which the young born mice are taken away from their mothers. As the pregnant mice were examined twice daily, at 9 a.m. and at 5 p.m., the longest time they remained with their mothers was 17 hours; only on Sundays they remained with their mothers for 24 hours. The majority of the newborn litters remained with their mothers for less than 17 hours. One litter of RIII mice was with its mother for over 24 hours, and both females of this litter developed mammary cancer.

As can be seen from Table II, mammary cancer appeared in the fostered RIII females at a slightly higher average age (9·3 months) than in non-fostered RIII females. This observation confirms the findings of Andervont and McEleney (1939*b*), and Andervont (1941), who noted in mice of C_3H strain that the amount of milk obtained from high-cancer strain mother influences the time of appearance and the incidence of mammary cancer in fostered high-cancer strain females.

Table II also presents data concerning the incidence of mammary cancer in the " S " low-cancer strain females fostered by RIII high-cancer strain mothers. The foster nursing of young " S " strain females by females of strain RIII increased the incidence of mammary cancer in " S " strain females from less than 1 per cent. to 29·6 per cent.—8 females out of 27 developed mammary cancer. Tumours arose in 8 out of 24 litters, i.e. in 33 per cent. of the litters. The 8 females which developed tumours came from 8 different litters which contained from one to four females.

The average age at which mammary cancer appeared in the fostered " S " strain females was lower (15·2 months) than the average age (19 months) at which tumours have on rare occasions been observed to appear in the non-fostered " S " strain females, but much higher than the average age of appearance of tumours in RIII females and in RIII females fostered by " S " strain mothers.

As can be seen, fostered mice with an inherited susceptibility to mammary cancer may not develop tumours if the influence contained in the mother's milk is lacking. A fostered resistant strain of mice in spite of its lack of inherited susceptibility may develop mammary cancer, though not to such a high degree as a non-fostered high-cancer strain of mice.

The present findings confirm the results of other workers reported previously, and prove that the milk of high-cancer strain RIII females contains an agent or agents playing an important part in the development of mammary cancer in mice.

SUMMARY.

The influence of foster nursing upon the occurrence of mammary cancer in two inbred strains of mice has been investigated.

Foster nursing of high-cancer strain RIII females by females of " S " low-cancer strain reduced the incidence of mammary cancer in RIII females from 83 per cent. at an average age of 8·5 months to 6·6 per cent. at an average age of 9·3 months.

Foster nursing of low-cancer strain " S " females by females of RIII strain increased the incidence of mammary cancer in " S " strain of mice from less than 1 per cent. at an average age of 19 months to 29·6 per cent. at an average age of 15·2 months.

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